

Science

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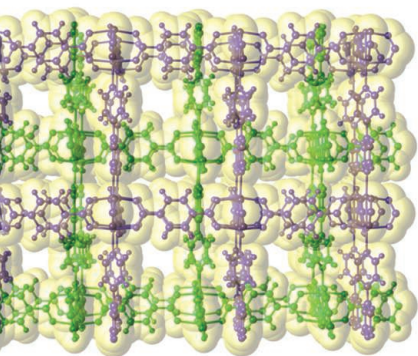
COVER

A yin-yang symbol superimposed on a scanning electron micrograph of a mouse tissue alveolar macrophage (diameter: ~18 micrometers). Macrophages are immune cells that mediate inflammation, but they often play protective roles as well. Several age-related chronic diseases—such as metabolic syndrome, cardiovascular disease, and neurodegenerative disease—have an inflammatory component. See the special section beginning on page 155.

Scanning electron microscope image: © Dennis Kunkel Microscopy, Inc.

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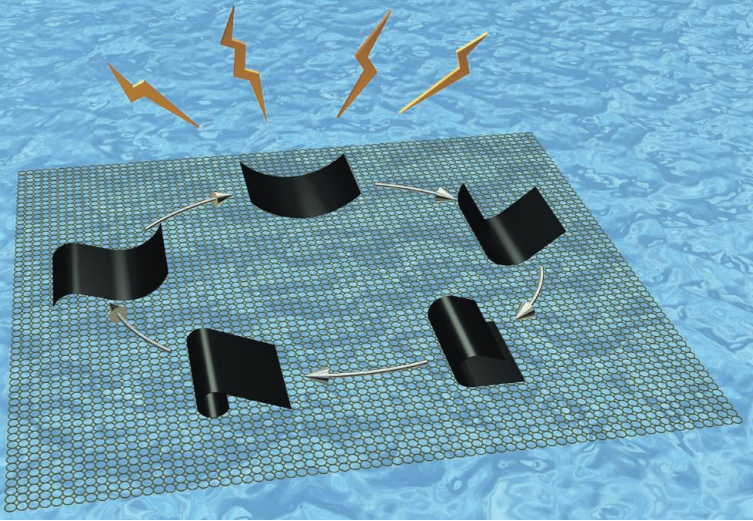
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<< Mini Mighty Muscle

Actuators—or artificial muscles—take electrical or chemical energy and convert it into mechanical force. Typically, actuators made from polymers can show large deformations, but cannot generate a lot of force. **Ma *et al.*** (p. 186; see the Perspective by **Kim and Kwon**) describe a polymer composite based on a modified polypyrrole that expands in response to water absorption. The composite was able to generate large stresses and forces, and offered a high work density approaching those of the best conducting polymer electrochemical actuators. Magnetic nanoparticles incorporated into polymer films were used to control the locomotion of the actuator.

Macrophage JNK in Metabolic Disease

Inflammation is thought to be an important driver of diet-induced obesity and insulin resistance. Proinflammatory, M1 phenotype macrophages and the c-jun NH₂ terminal kinases (JNK) are central players in this process. But whether JNK expression is specifically required inside macrophages is unclear. In mice containing a macrophage-specific deletion in both *Jnk1* and *Jnk2*, **Han *et al.*** (p. 218, published online 6 December; see the Perspective by **Ferrante Jr.**) found that the mice were protected against many of the diet-induced metabolic changes, including insulin resistance, despite similar weight gain as control mice on a high-fat diet. This protection was associated with a decrease in the presence of M1 macrophages in adipose tissue.

Tracking Quantum Evolution

The actual process of measuring a quantum system has an effect on the result making the outcome unpredictable. Using a superconducting qubit placed in a microwave cavity, **Hatridge *et al.*** (p. 178) found that a series of partial measurements on a quantum system left the system in a pure state. Looking at the record of the actual measurements allowed the final state of a superconducting-based quantum system to be determined accurately. Such control is crucial for achieving full feedback control of a general quantum system.

Ribosomal Rotaxane?

The ribosome is an extraordinarily sophisticated molecular machine, assembling amino acids into proteins based on the precise sequence dictated by messenger RNA. **Lewandowski *et al.*** (p. 189) have now taken a step toward the preparation of a stripped-down synthetic ribosome analog. Their machine comprises a rotaxane—a ring threaded

on a rod—in which the ring bears a pendant thiol that can pluck amino acids off the rod; the terminal nitrogen then wraps around to form a peptide bond and liberate the thiol for further reaction. The system was able to link three amino acids in order from the preassembled rod.

Pupfish Speciation

Evolution moves along phenotypic trajectories that can be visualized as a topographic landscape of multiple peaks of relatively high-fitness and low-fitness valleys. **Martin and Wainwright** (p. 208) examined the adaptive landscape of three species of *Cyprinodon* pupfishes. These species represent a recent adaptive radiation, each having moved into a difference niche within their specialized environment. Examining replicate hybrid transplants relative to parental types in high- and low-density enclosures, the authors recovered the specialist parental phenotypes and observed higher survival and growth. Thus, high density can drive multiple fitness peaks during the early stages of adaptive radiation.

A Varied Bouquet

Pollinators display innate attractions to odor, but can also learn to associate odor with a nectar reward. **Riffell *et al.*** (p. 200, published online

6 December; see the Perspective by **Knaden and Hansson**) characterized the odor profile for flowers to which hawkmoths are innately attracted and found that the majority contain a distinct chemical profile, which is uniquely represented on their olfactory lobe. The moths could also be trained to associate nonattractive odors with a reward and thus learn novel odor attractions. Though learning altered neurons within the antennal lobe, the innate preferences were not changed.

Optimizing Carbon Nanotubes

Shorter carbon nanotubes are easier to make, but, when assembled into fibers, the resulting fiber properties are much poorer than might be predicted by theory. Conversely, longer carbon nanotubes have much better properties but are harder to process. **Behabtu *et al.*** (p. 182) combined the best of both worlds through scalable wet spinning method, in which they dissolved longer carbon nanotubes and then spun them into fibers that showed excellent strength, stiffness, and thermal conductivity.

Stress Protector

During prolonged fasting, the oxidation of fatty acids leads to increased accumulation of D-β-hydroxybutyrate (βOHB) in the bloodstream. Such increased concentrations of βOHB inhibit class I histone deacetylases. Histone acetylation in turn influences transcriptional activity at various genes. **Shimazu *et al.*** (p. 211, published online 6 December; see the Perspective by **Sassone-Corsi**) found that among the genes showing increased transcription in animals treated with high concentrations of βOHB were two genes implicated in cellular responses to oxidative stress. When treated ahead of time with βOHB, mice were protected from the toxic effects of the oxidative stress causing poison paraquat.





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Funding Innovative Science

IT IS A WELL-KNOWN PROBLEM: A JUNIOR RESEARCH GROUP LEADER MUST SOMEHOW COMPETE against the seniors, who have larger laboratories, good funding, and clout with the journals. Furthermore, in the normal grant system, preliminary data requirements make it hard to start new directions in research. Beginning scientists must build on their postdoctoral work, which forces them to continue along already-trodden paths. Once a laboratory has been established, it is reasonable for the reviewers of competitive grant applications to look for evidence of an investigator's likely success in the form of "preliminary results." But beginning group leaders should be judged only by their demonstrated excellence and their creativity in finding new directions. Such a change would greatly stimulate innovation.

I experienced the advantages of such a system 20 years ago, when moving to Germany from a postdoctoral position in the United States. A hierarchical system that emphasized seniority was rampant in Europe, and independent research positions for younger scientists were few. But I was lucky in joining the European Molecular Biology Laboratory (EMBL) in Heidelberg, which was exploring alternative ways to organize science, with the aim of promoting innovation and research excellence.

The EMBL had created a group leader system in which, apart from a few senior scientists to provide some stability, new researchers were directly funded for up to 9 years to do as they pleased, before being required to move on to a senior job elsewhere. This model was a success in large part because this type of funding encouraged a focus on innovation, but also because it provided a separate funding stream in Europe for starting scientists. The EMBL was not alone in this endeavor, as analogous thinking was beginning to take hold at other European institutions and funding agencies. But in 2007, the European system moved an important step further with the introduction of the European Research Council (ERC). The ERC currently runs a pan-European competition that in 2012 funded 536 proposals after receiving more than 4700 applications from beginning group leaders, each for 5 years for as much as 1.5 million euros per year. This grant program is specifically targeted at providing additional opportunities for young investigators who are "making the transition from working under a supervisor to being independent researchers in their own right." A crucial aspect of the ERC is that the reviewing criteria specifically focus on novelty, interdisciplinarity, and high-risk/high-gain research.* The ERC runs other competitions to fund established investigators.

In the United States, the New Innovator Awards from the National Institutes of Health (NIH) are similar to the ERC junior grants in seeking innovation and explicitly not requiring preliminary data. However, there are far too few of them. Although a *Science* Editorial called for 500 of these grants in 2009,[†] only 51 were awarded in 2012. I suggest that NIH move to a model where all starting principal investigators are funded through a New Innovator Award type of program before they compete in the normal system. The new idea here is that a screen for excellence and innovation should be the only way in which new investigators get funded, through a separate funding stream, with no requirement for preliminary data.

In my own field of the biological sciences, the molecular biology revolution with its associated cataloging of gene function is approaching maturity. To understand how cells and organisms function, biology must now branch out into new areas, incorporating physics, chemistry, and engineering. No one knows the right way ahead, but each new laboratory should be a small experiment in that direction. Experience demonstrates that innovation in science mainly comes from the young. Only by providing our new group leaders with real freedom to maneuver can we sow enough seeds to find the right way ahead.

— Anthony A. Hyman

10.1126/science.1234741



*<http://erc.europa.eu/starting-grants>. †B. Alberts, *Science* **326**, 1163 (2009).

CHEMISTRY

Acid to Acid

Hydration of sulfur trioxide (SO_3) to sulfuric acid (H_2SO_4) in Earth's atmosphere gives rise to acid rain as well as particle formation. A sound mechanistic understanding of this reaction is thus central to the chemistry of climate and pollution mitigation. Although the necessary bonding rearrangements appear straightforward—S-O bond formation accompanied by proton transfer from water to any of the other oxygen atoms—the direct bimolecular reaction is in fact extremely slow. Theoretical and experimental studies have demonstrated that the assistance of a second, catalytic, water molecule is crucial to facilitate the proton transfer. Torrent-Sucarrat *et al.* now report, on the basis of extensive theoretical calculations, that a second sulfuric acid molecule ought to be an even more potent catalyst; in other words, the reaction is autocatalytic. The authors combine electronic structure calculations with a transition-state theory framework to predict rate-constant ratios, and then estimate the prevalence of this pathway depending on the relative background concentrations of water and sulfuric acid. The sulfuric acid-to-water ratio is too low for significant autocatalysis in the troposphere, but the pathway could play a pivotal role in the stratosphere as well as in aerosols, and perhaps in the atmosphere of Venus. — JSY

J. Am. Chem. Soc. **134**, 20632 (2012).

ECOLOGY

Vital Variation

It is increasingly recognized that we are in the midst of a sixth mass extinction due to a suite of anthropogenic activities. How different species respond to these changes is varied. González-Suárez and Revilla ask whether there are clear patterns in susceptibility to extinction among mammals, using the database panTHERIA, which consists of data on over 4400 mammalian species. They examined morphological, ecological, and life-history traits as they relate to the threat

of extinction. As might be expected, species with lower densities and slower rates of reproduction and maturation, such as larger mammals, experienced a greater risk of decline. However, greater variation in traits such as body size and litter size, among others, was associated with reduced risk of extinction, regardless of body size. This suggests that species that maintain variability at the population level may be better able to adapt to changing environmental conditions. These results can inform conservation efforts across an array of species and emphasize the importance of protecting natural variation in all its forms. — SNV

Ecol. Lett. 10.1111/ele.12035 (2012).

trapped single atoms or ions should be more stable because the atoms are vacuum-packed and do not interact. However, these tend to be relatively large and power-hungry. Jau *et al.* have developed a miniature atomic clock based on trapped Yb ions that has size and power requirements similar to those of existing CSAC technology but also offers to match the long-term stability expected of the much larger trapping systems. — ISO

Appl. Phys. Lett. **101**, 253518 (2012).

PHYSICS

Classifying Topological Phases

One of the most exciting developments in condensed-matter physics over the past few years has been the prediction and discovery of new topological phases of matter. The most interesting of these, topological insulators, are characterized by surface states robust to perturbations that preserve time reversal symmetry (TRS). Recently, other symmetries, such as spatial inversion, have been demonstrated to be protective of topological states. Slager *et al.* have greatly expanded the theoretical framework for identifying new topological states in non-interacting fermionic systems by classifying states protected by different space group symmetries of the underlying crystal lattice. They found two broad classes of such phases, one where the protection comes from both TRS and space group symmetry, and another which is protected by lattice symmetry alone and would be considered topologically trivial under the TRS-based classification system. This led to

PHYSICS

Shrinking Atomic Clocks

Atomic clocks housed in national laboratories around the world provide a precise time signal on which our present high-technology lives depend. Precise synchronization allows for colossal amounts of data to be reliably transferred around networks of optic fibers at ever-increasing rates, as well as providing accurate navigation systems. With increasing demand for mobile devices, there is a requirement for these atom-based time pieces to shrink. Present chip-scale atomic-clock (CSAC) devices based on alkali atoms in a buffer gas are lightweight (<50 g) and consume little power (<150 mW) but tend to show frequency drift over time that requires frequent recalibration. Clocks based on



the identification of 18 distinct phases in two dimensions and at least 70 in three dimensions. It is expected that the classification will guide future experimental efforts and inform the study of topological matter in the presence of interactions. — JS

Nat. Phys. 10.1038/NPHYS2513 (2012).

ECOLOGY

Microbial Conversion

Conversion of tropical forest to pasture has been widespread in the Amazon Basin in recent decades, a process that has been accompanied by a loss of diversity of flora and fauna and often by a reduction in soil fertility. However, how conversion affects soil microorganisms is largely unknown. Using DNA sequencing techniques, Rodrigues *et al.* compared the bacterial composition of forest and pasture soils at a site in Rondonia, Brazil. In pasture soils, there was a reduction in the diversity of taxa in the phylum Acidobacteria, organisms that are sensitive to increases in pH and soil carbon content, both of which occur after forest clearance. On the other hand, there was an increase in diversity within the phylum Firmicutes, which are tolerant of desiccation and greater extremes of temperature. At individual sample sites, the alpha diversity (number of taxa) of bacteria was higher in the pasture soils than in forest soils, but the beta diversity (variation between sites) of the pasture soils was significantly lower than in the forest. The lower beta diversity of the pasture soils implies a biotic homogenization of the soil microbiota after the conversion to pasture, and an eventual loss of overall diversity despite local increases in alpha diversity. — AMS

Proc. Natl. Acad. Sci. U.S.A. 10.1073/pnas.1220608110 (2012).

BIOPHYSICS

Dying to Survive

Bacteria grown on surfaces often form dense communities, often with complex three-dimensional wrinkled structures, called biofilms. These are often problematic—for example, in industrial and medical settings—but can also be harnessed for uses such as waste remediation. Asally *et al.* showed that in *Bacillus subtilis*, wrinkling is caused by localized cell death that spatially focuses mechanical forces and may be a community-level stress response. Wilking *et al.*, also working with *B. subtilis*,

showed that the wrinkles make up the top of a network of channels that provide a system for enhanced transport of nutrients. As biofilms grow, nutrients are consumed by cells on the periphery, leaving bacteria in the center facing nutrient depletion. Such channels provide a conduit for nutrient flow and are most highly connected near the center of the biofilm. Flow through the channels was driven by spatial variation in evaporation and also by factors such as osmotic pressure gradients. Channels are internalized as the biofilm ages; however, the network remains permeable even in the late stages of biofilm growth, which suggests that the channels may be physiologically relevant throughout the biofilm life cycle. — VV

Proc. Natl. Acad. Sci. U.S.A. 109, 18891; 10.1073.pnas.1216376110 (2012).

BIOENGINEERING

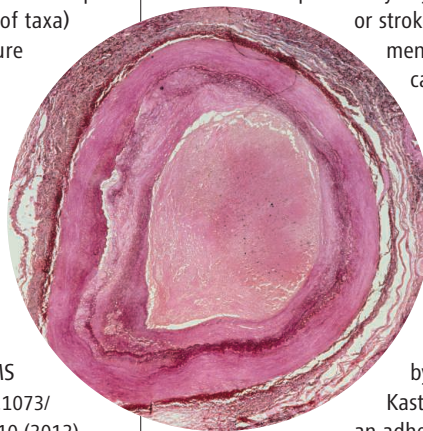
Vascular Glue

Atherosclerosis is characterized by the accumulation of plaque—lipid-laden inflammatory cells surrounded by a fibrous cap—in the inner lining of blood vessels. In so-called vulnerable plaques, the fibrous cap is at high risk of rupture, an event that results in blood clots that can potentially trigger a heart attack or stroke. The develop-

ment of strategies that can stabilize and/or heal vulnerable plaques is a major goal of cardiovascular research.

Inspired by the properties of underwater adhesives secreted by marine mussels, Kastrup *et al.* designed an adhesive hydrogel that can be painted on atherosclerotic plaques, with the idea that this “glue” might be useful both for localized deposition of drugs and for strengthening the fibrous cap to prevent its rupture. In a proof-of-concept study, gel containing dexamethasone, an anti-inflammatory drug, was applied via a catheter to inflamed atherosclerotic plaques in the carotid arteries of mice. The gel remained adherent for over a month, and the plaque in the gel-treated mice displayed a thicker fibrous cap and a 25% reduction in a marker of inflammatory cells as compared with plaques in control mice. — PAK

Proc. Natl. Acad. Sci. U.S.A. 10.1073/pnas.1217972110 (2012).



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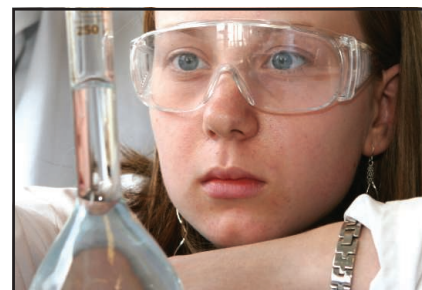
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Deepwater Horizon

with oil giant BP, which was using the rig. Transocean will also provide \$150 million to the U.S. National Fish and Wildlife Foundation for ecological restoration projects along the Gulf. NFWF has already received \$2.4 billion from the earlier BP settlement. BP still faces pollution penalties that could total \$5 billion to more than \$20 billion.

<http://scim.ag/Transocean>

Washington, D.C. 4

Stem Cell Legal Battle Ends

The U.S. Supreme Court has rejected a case challenging government-funded research on human embryonic stem cells (hESCs). The decision ends a court battle that has cast a shadow over hESC studies for more than 3 years.

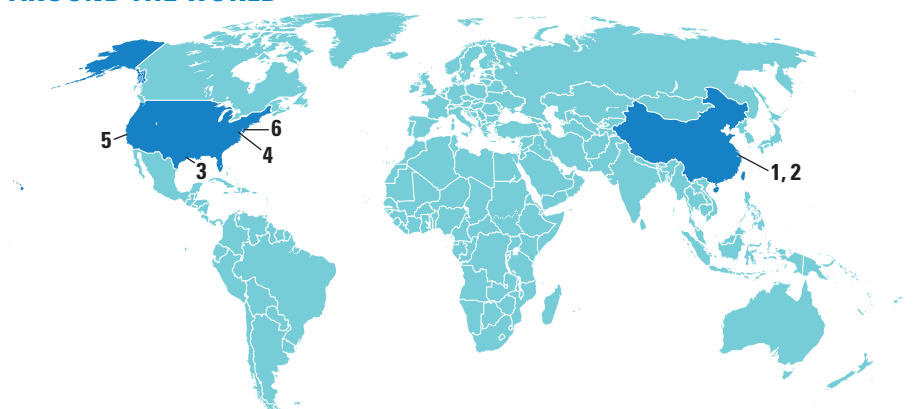
In *Sherley v. Sebelius*, two researchers argued that new National Institutes of Health (NIH) guidelines easing George W. Bush-era restrictions on hESC research violated a law banning federal funds for research on embryos. The plaintiffs won a preliminary injunction in August 2010 that briefly shut down NIH-funded hESC research. A trial court and an appeals court later ruled in favor of NIH. In October 2012, the plaintiffs appealed to the Supreme Court, which this week declined to review the case.

Mountain View, California 5

BGI-Shenzhen Cleared to Take Over Complete Genomics

A review panel has dismissed concerns that a merger between the Chinese sequencing dynamo BGI-Shenzhen (*Science*, 3 February 2012, p. 516) and a struggling California company, Complete Genomics Inc., would threaten U.S. security. In September, BGI offered \$117.6 million for control of Complete Genomics and promised to invest \$30 million in cash to rejuvenate the firm. Founded in 2005, Complete Genomics uses proprietary methods to sequence entire human genomes with accuracy. The company has been losing money since its launch, however, and seemed headed for collapse in 2013. It accepted the lifesaving offer from BGI, but the deal ran into opposition. Several critics—including a U.S.

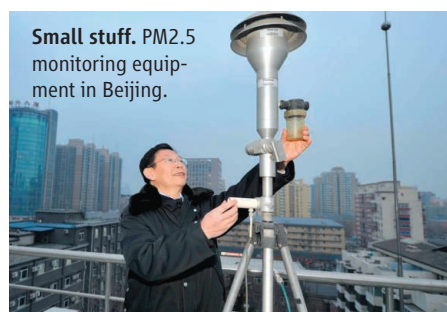
AROUND THE WORLD



Shanghai, China 1

Clearing the Air on Particulates

Following public pressure for greater transparency in pollution reporting, 74 Chinese cities began measuring concentrations of harmful particulate matter less than 2.5 micrometers in diameter (PM_{2.5}) on 1 January. Air pollution has become a touchy subject in China; the Twitter feed @BeijingAir, run by the U.S. Embassy in



Small stuff. PM_{2.5} monitoring equipment in Beijing.

Beijing, provides hourly PM_{2.5} readings that provoked protests from Chinese officials in 2009. @BeijingAir helped prompt the Chinese government to release its own PM_{2.5} readings for Beijing last January, but those often differ from the U.S. Embassy's readings, both in absolute measurements and in how the figures are interpreted.

Chinese government descriptions "significantly understate the severity of pollution," says Steven Q. Andrews, an independent environmental consultant in Beijing who has studied the issue. Days described by the U.S. Embassy as "very polluted" or "hazardous" have been called "good" or "slightly polluted" by the Chinese government in the past, he says. The new pollution readings appear in Chinese on the China National Environmental Monitoring Center's Web site: www.cnemc.cn.

Shanghai, China 2

Toughening Up on Academic Fraud

A Chinese government regulation toughening penalties for academic fraud took effect on 1 January. Cases of fraud are rife in China and include fabricated data and padding resumes with honors that a scholar never earned. A key provision of the new regulation stipulates that academic degrees will be revoked if misconduct involving graduate or postgraduate thesis work is discovered retroactively.

Cong Cao, a scholar of Chinese science policy at the University of Nottingham in the United Kingdom, calls the directive a step forward but says implementation could be spotty: "The litmus test is whether high-profile scientists and administrators will be punished for misconduct." He fears that "the regulation will end up being used to punish ordinary students and faculty members," he adds.

Houston, Texas 3

Transocean Owes \$1 Billion For Clean Water Act Violations

Gulf of Mexico science and restoration will get another chunk of cash from a record settlement with the U.S. government of charges related to the 2010 *Deepwater Horizon* oil spill. Transocean Deepwater Inc., the company that owned the doomed drilling rig, agreed on 3 January to pay \$1.4 billion in civil and criminal fines and penalties. A record \$1 billion is for violations of the Clean Water Act, 80% of which will be dedicated to economic and ecological restoration projects along the Gulf Coast. In addition, the U.S. National Academy of Sciences will get \$150 million to help fund a \$500 million, 30-year Gulf Coast research program created last November under a criminal settlement

maker of sequencing machines, Illumina of San Diego, California—warned that the deal might give China technology of key economic or military value (such as bio-weapons manufacturing). In late December, however, the Committee on Foreign Investment in the United States ruled out such worries. This week, the deal emerged from a second waiting period that allows for an anticompetitive risk review and now seems cleared for takeoff.

Princeton, New Jersey 6

Princeton, South Korea Collaborate on Fusion Reactor

The Princeton Plasma Physics Laboratory (PPPL), the United States' foremost fusion lab, is to collaborate with researchers in South Korea to design a next-generation fusion power demonstration plant that could generate electricity sometime after 2030. Fusion reactors generate energy by fusing hydrogen isotopes—but no fusion reactor has yet produced more energy than required to keep it running. The United States and South Korea are partners—along with China, the European Union, India, Japan, and Russia—in ITER, an international project to build a reactor that can produce excess energy. ITER's target is to produce 500 megawatts (MW) for 500 seconds at a time. Scientists at PPPL are preparing a work plan this month for the collaboration, which initially will run for 6 months. K-DEMO will be roughly the same size as ITER but it will produce 1000 MW and run for several weeks continuously. Rather than constructing an international demonstration reactor, many of the partners are considering building their own demonstration plants, including China, Japan, and India—and now South Korea looks set to join the race.

NEWSMAKERS

'Third Kingdom' Discoverer Dies

On 30 December, microbiologist **Carl Woese**, who revolutionized microbiology through his studies of single-celled microbes called archaea, died at age 84 of complications from pancreatic cancer. When he began studying archaea, a group that includes methanogens and heat-loving thermophiles, biology textbooks contained only



Woese

two main branches on the tree of life: bacteria and eukaryotes. Archaea were considered evolutionary offshoots of bacteria. But in 1977, by painstakingly hand-sequencing the ribosomal RNA of microbes, Woese demonstrated that archaea are as genetically distinct from bacteria as plants and animals are and that they merit their own branch. The discovery was controversial, upending traditional views of the evolution of life and taking years to gain widespread acceptance. Today, however, "nearly all biologists and an increasing number of physicians have awarded Woese an honorary Nobel Prize," says Alan Weiner, a professor of biochemistry at the University of Washington, Seattle.

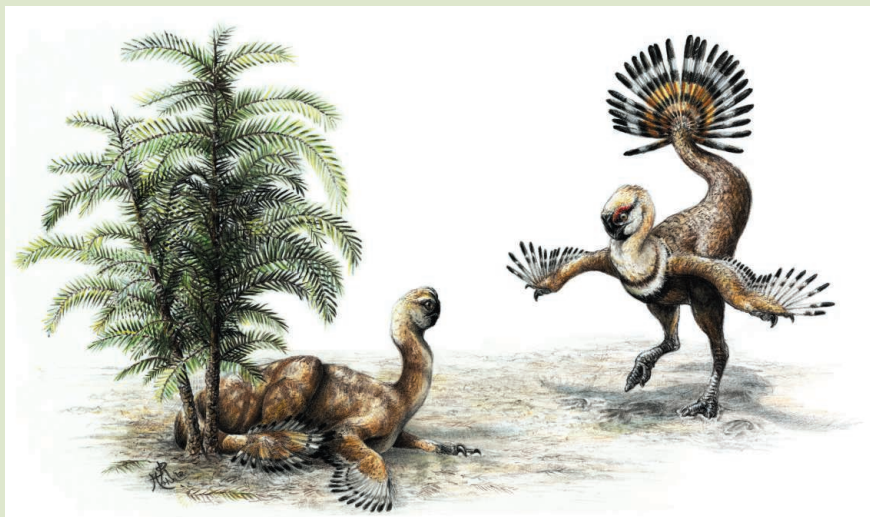
Pioneering Neurobiologist Dies

The world's first Nobel Prize-winning centenarian—and one of its great scientific personalities—died on 30 December. **Rita Levi-Montalcini**, who was 103, was awarded the Nobel Prize in physiology or medicine in 1986 for her discovery of nerve growth fac-

tor. She spent the early years of her career in hiding in wartime Italy, studying the development of chick embryos in a makeshift lab set up in her bedroom after the Fascist government of Italy banned Jews from university posts. After the war, she worked for more than 2 decades at Washington University in St. Louis, where she made her seminal discoveries about how growth factors influence development and disease. In the 1960s, she began to divide her time between St. Louis and Rome, where she established a lab as well. After her nominal retirement in 1977, she returned to Italy full time. She remained active in the lab well past her 100th birthday and served until her death as president of the European Brain Research Institute in Rome, which she helped found, and at the Levi-Montalcini Foundation, which supports scientific education for girls and women in Africa. >>



Levi-Montalcini



Dinos Shook Their Tail Feathers

A close look at 150-million-year-old fossils of a group of bipedal dinosaurs known as oviraptors suggests that many of them shook their muscular, feather-adorned tails to gain attention during courtship. The oviraptor's relatively stubby tail, scientists found, was muscular and flexible at its base but rigid at its tip, where the last half-dozen or so vertebrae are either fused together into a bladelike structure or so tightly arranged that they're inflexible. Some early fossils from the group included large feathers either attached to the last few tail vertebrae or preserved nearby. Fossils from later species had no preserved feathers, but had the same arrangement of tail vertebrae and the muscles used to control their movements, researchers revealed last week in *Acta Palaeontologica Polonica*. The most likely use for a feather-tipped tail is for courtship display (pictured: artist's reconstruction of a male oviraptor *Ingenia yanshini* flirting with a potential mate).

>>NEWSMAKERS

Eleven Engineers Win NAE Awards

The U.S. National Academy of Engineering announced last week that it will honor three teams for notable achievements in telecommunications, bioengineering, and engineering education at a gala in Washington, D.C., on 19 February. Each team will share a \$500,000 prize.



Martin Cooper, Joel S. Engel, Richard H. Frenkiel, Thomas Haug, and Yoshihisa Okumura will receive the Charles Stark Draper Prize “for their pioneering contributions to the world’s first cellular telephone networks, systems, and standards.” **Rangaswamy**

Srinivasan, James J. Wynne,

and **Samuel E. Blum** will receive the Fritz J. and Dolores H. Russ Prize “for the development of laser ablative photodecomposition, enabling LASIK and PRK

eye surgery.” And **Richard K. Miller, David V. Kerns Jr., and Sherra E. Kerns** will receive the Bernard M. Gordon Prize “for guiding the creation of Olin College and its student-centered approach to developing effective engineering leaders.” <http://scim.ag/NAEprize>



FINDINGS

Hope for Coral Reefs

Global warming is expected to devastate coral reefs worldwide—but scientists have taken a step toward identifying the genetic mechanisms that give some corals a natural resilience to thermal stress. Coral reef ecologist Daniel Barshis and colleagues at Stanford University in Palo Alto, California, collected samples of the thermally tolerant *Acropora hyacinthus* in reefs in two pools at Ofu Island, American Samoa; one pool had highly variable temperatures, topping 34°C in summer, whereas the other was more moderately variable. The team subjected the samples to laboratory thermal stress experiments while monitoring the activity of a range of genes.

The researchers identified 60 genes that were normally more active in the corals from the highly variable pool—but became more active in corals from the moderately variable pool when water temperatures rose. The higher gene expression under normal conditions—called “frontloading”—may prepare these



BY THE NUMBERS

830 million Tons of carbon dioxide (2% of global emissions) that the world’s telecommunications infrastructure produces each year, according to a study published on 2 January in *Environmental Science & Technology*.

100 billion Planets in our Milky Way galaxy (averaging one per star), according to a new estimate in *The Astrophysical Journal* based on M dwarf systems, the most common type of system in the Milky Way.

resilient corals for periodic hot water, the team reported online this week in the *Proceedings of the National Academy of Sciences*. <http://scim.ag/Ofureef>

Drug Restores Hearing in Mice

A drug applied to the ears of mice deafened by ear-splitting noise can restore some hearing in the animals. The compound, which blocks the action of a key developmental protein called Notch, allows the sound-sensing inner ear cells damaged by the noise to regrow. Although birds and fish can regenerate these so-called hair cells, mammals can’t do so. Because noise, antibiotics, and other insults can harm hair cells, researchers have looked for ways to reactivate that latent regenerative potential. In 2005, scientists used gene therapy to prompt the growth of hair cells in adult guinea pigs, which also restored some hearing. However, the drug approach would potentially be much simpler, says Albert Edge, a stem cell biologist at the Massachusetts Eye and Ear Infirmary in Boston. He and his colleagues describe their results with the drug, originally developed as a potential Alzheimer’s treatment, in the 10 January issue of *Neuron*. It is not ready yet to try in humans, Edge says, “but it’s a foot in the door.”

Random Sample

Not Slippery When Wet

The pale wrinkles that adorn fingertips after an extended soaking may be unsightly, but they serve a purpose: They help us get a stronger grip on slippery objects.

Long thought to be caused by osmosis-induced swelling in the outer layer of skin, the wrinkles are in fact produced by the autonomic nervous system, recent experiments indicate. But the purpose of the puckering—which occurs on hair-free skin of the hands, feet, and toes but nowhere else on the body—hasn’t been clear, says Tom Smulders, an evolutionary biologist at Newcastle University in the United Kingdom.

In 2011, a team of neuroscientists suggested that the wrinkles served to enhance our grip on wet or submerged objects, just as treads on tires help improve traction on wet roads. “That seemed like a clever hypothesis that would be easy to test,” Smulders says.

So he and his colleagues designed a test in which volunteers picked up 45 submerged objects—such as glass marbles and lead fishing weights—from a bin one at a time, passed them to their other hand through a postage stamp-sized hole in a barrier, and then dropped them through another hole into a box. When test subjects had wrinkly fingertips—induced by soaking their hands in 40°C water for 30 minutes—they completed the task about 12% faster than they did when their fingers hadn’t been soaked, the team reported on 8 January in *Biology Letters*. When performing the same task with dry objects, wrinkly fingertips didn’t provide an advantage.



Science LIVE

Join us on Thursday, 17 January, at 3 p.m. EST for a live chat with experts about **what it may take to phase out greenhouse gas emissions**. <http://scim.ag/science-live>

U.S. BUDGET

Sequestration Takes Aim At Federal Science Spending

The battle over the fiscal cliff is far from over for U.S. scientists. Last week's passage of the American Taxpayers Relief Act may have settled the fight between Democrats and Republicans over changes in the country's tax code. But it simply provides a 2-month reprieve for resolving the spending side of the crisis, a hiatus that leaves the status of thousands of federal programs up in the air.

In the next few months, Congress and the Obama administration must decide whether to prevent a blunt ax known as sequestration from lopping tens of billions of dollars from all discretionary government spending in the current fiscal year, including research. They also need to agree on how to extend the nation's debt ceiling and deal with a freeze in current spending levels, as well as take a first shot at the president's budget request for 2014.

Those debates will occur under a looming 1 March deadline, the date the sequestration ax is now set to fall. Last fall, the White House projected that sequestration would result in an 8.2% cut for civilian agencies like the National Institutes of Health and the National Science Foundation, resulting in thousands of fewer grants, less support for graduate students, and the likely cancellation of some activities. The delay gives U.S. scientists more time to make the case to Congress that academic research should be protected from such deep cuts because it's essential for reviving the economy. To succeed, however, the scientific community must blunt the arguments of fiscal conservatives who believe that the economy won't recover until the country stops spending money it doesn't have and that research needs to be downsized along with the rest of the federal government.

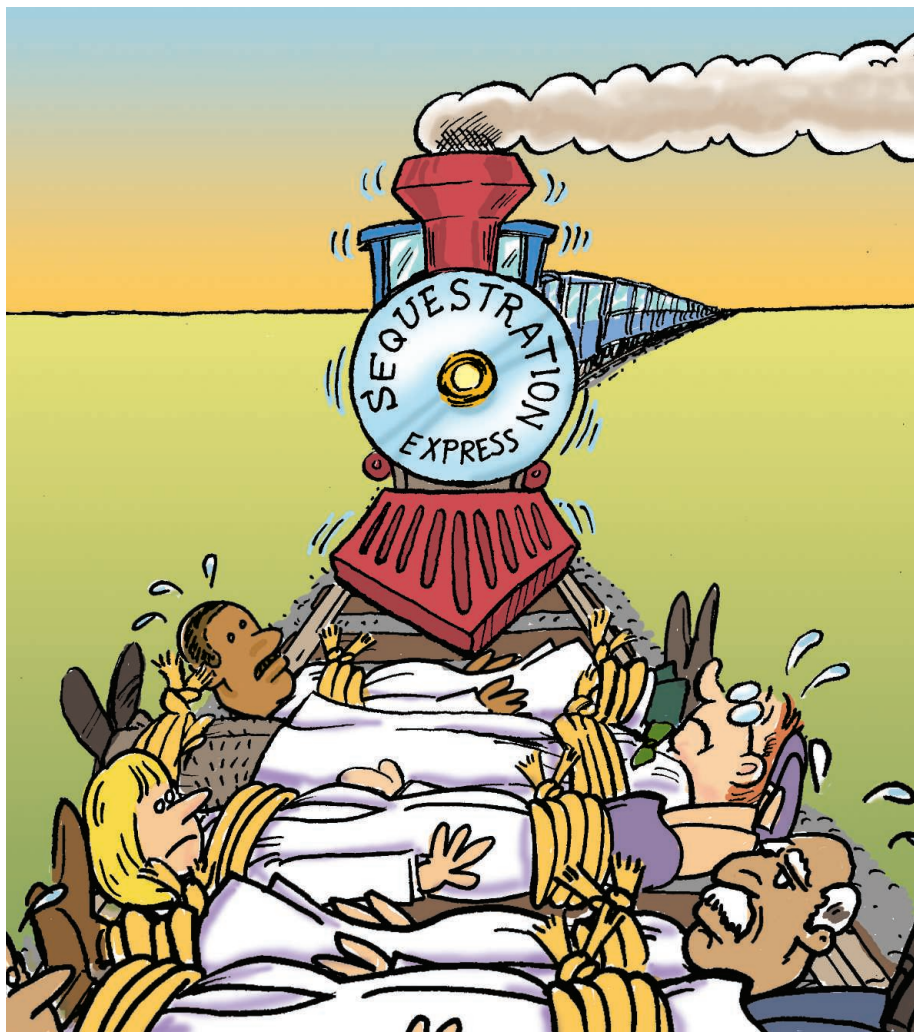
How did we get here? In August 2011, Congress passed the Budget Control Act, under which both sides agreed to make \$1 trillion in unspecified spending cuts over the next decade. The law also created a commission to recommend additional cuts and revenue increases, with the provision that \$1.2 trillion in across-the-board cuts would take effect automatically on 2 January 2013

if Congress failed to adopt the commission's recommendations. It didn't, and policy-makers have spent the past 12 months in bitter and, to date, fruitless negotiations.

The new law delays the start of sequestration for 2 months. It also pares \$24 billion from the original \$109 billion in cuts scheduled to take effect for the remainder of the fiscal year, which ends on 30 September. But it does so by taking \$4 billion from the pot of money available this year—and \$8 billion from 2014—for legislators to allocate as they wish. Those cuts will make it even harder for Congress to protect any specific programs as it determines the fate

of the overall 2013 budget, now under a continuing resolution that freezes spending at 2012 levels until 27 March. A government-wide shutdown could occur if Congress does not complete work on the current budget or extend the temporary spending measure.

Congress will also be debating whether to give the administration the authority to borrow more money as the nation bumps up against a \$16.5 trillion debt ceiling. In this case, inaction could trigger a government default and wreak havoc on global financial markets. Finally, President Barack Obama will submit his 2014 budget request to Congress sometime this winter. (He's expected to miss the usual date of early February.) The request will spell out the president's spending priorities for all federal activities. And if Obama's previous statements are any guide, his 2014 budget is likely to request increases for the physical sciences, clean energy technologies, and training the next generation of scientists. Some of the increases could



CREDIT: (ILLUSTRATION) JOE SUTLIFF/WWW.CDAD.COM/JOE

be premised on raising revenues from various sources. But there will likely be offsetting cuts in other programs, as the president abides by the lower overall spending ceiling in the new law.

So sequestration, which was supposed to be unthinkable, is now, perhaps more than ever, on the minds of science lobbyists. “[We remain] very concerned about the impact the indiscriminate spending cuts required by the sequester will have on scientific research if it is not repealed completely,” said The Science Coalition, an advocacy group for academic research. “The two-month delay and lower caps on discretionary spending only perpetuate the negative impact on science funding. We

urge members of the 113th Congress to act quickly to find deficit reduction solutions that do not disproportionately affect scientific research.”

The problem, of course, is that Congress and the White House have so far failed to find any acceptable alternatives. After the collapse of negotiations between Obama and House of Representatives Speaker John Boehner (R-OH), Vice President Joe Biden and Senate Minority Leader Mitch McConnell (R-KY) finally struck a deal for changes in the U.S. tax code that cleared the Senate and the House within a 21-hour span on New Year's Day.

The administration claims that the new tax rates will generate \$660 billion in addi-

tional revenue over 10 years, while the nonpartisan Congressional Budget Office (CBO) says it will actually cost the government \$4 trillion. The disparity stems from the use of different baselines: Obama is measuring the new law against what existed in 2012, whereas CBO is comparing it to what would have occurred had all the tax breaks expired and sequestration kicked in. In either case, however, it's clear that the new law fails to dent the current \$1.3-trillion-a-year deficit.

That's because it ignores the other two legs of deficit reduction—reining in entitlement programs like Medicare and reducing annual spending. House Republicans initially threatened to balk at the tax deal after

GLOBAL WARMING

Climate Study Highlights Wedge Issue

Eight years ago, Steven Davis was a 26-year-old graduate student when he heard an energy scientist give an inspiring talk about tackling the global climate challenge. The speaker, Robert Socolow of Princeton University, had just co-authored a plan for “Stabilization Wedges: Solving the Climate Problem for the Next 50 Years with Current Technologies,” as the title put it (*Science*, 13 August 2004, p. 968). Humanity could stabilize rapidly rising annual carbon emissions at 2004 levels by 2054, Socolow argued, if it embarked on seven massive campaigns that would each prevent 25 billion tons of carbon emissions over 5 decades; the options included building a fleet of nuclear reactors and ending tropical deforestation. Socolow and Princeton ecologist Stephen Pacala dubbed each campaign a “wedge,” after the angular shapes formed on a graph used to

illustrate the concept (see figure).

Now, Davis, an earth systems scientist on the faculty at University of California, Irvine, has offered a provocative update to the wedges saga. In a paper published this week, he and three colleagues call for a more audacious plan, and calculate that at least a whopping 19 wedges—and perhaps as many as 31—will be needed to stabilize and then phase out carbon pollution. Beyond the 2004 plan's emphasis on expanding the use of current technologies, “fundamental and disruptive transformation of the global energy system” is needed to avoid devastating climate change, the authors warn in *Environmental Research Letters*.

The original wedges paper was very influential, shaping policy in President George W. Bush's administration and getting cited by scientists more than 1200 times. In part, that's because it offered hope, Davis

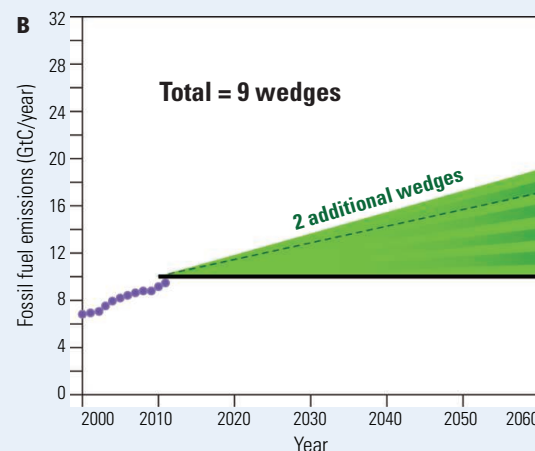
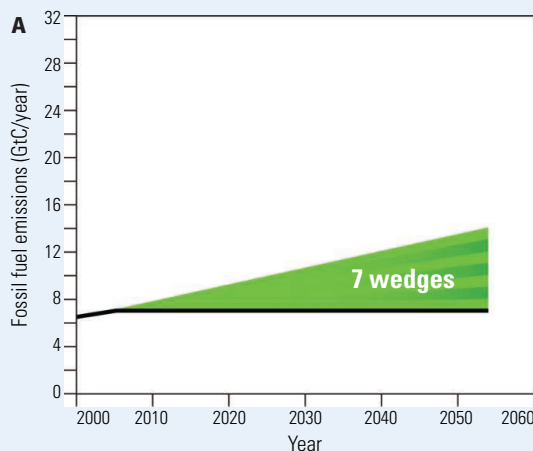
says. “Each wedge is incredibly ambitious, but by breaking up the problem it made me feel we could do it.”

Yet the iconic paper also proved controversial. Critics argued that Pacala and Socolow proposed too few wedges to achieve their stated goals, and gave short shrift to the need for fundamental research into breakthrough energy technologies. Subsequent findings suggested that their initial target—capping atmospheric concentrations of carbon dioxide at 500 parts per million by midcentury—wasn't aggressive enough to avoid dangerous warming.

Davis's sobering reassessment utilizes new emissions data, better understanding of climate risks, and more refined computer models of the global climate cycle. It also reflects the failure of governments to enact policies to limit global emissions. “We haven't acted and things are getting worse,” he says.

Everywhere Davis and his co-authors looked, they found more wedges needed. The 2004 proposal, for instance, estimated it would

New angle. (A) The 2004 “wedges” paper estimated it would take seven major efforts to reduce global carbon emissions to stabilize annual emissions by 2054. (B) A new study raises the number to nine wedges by 2060, in part because emissions have risen faster than expected (dotted line).



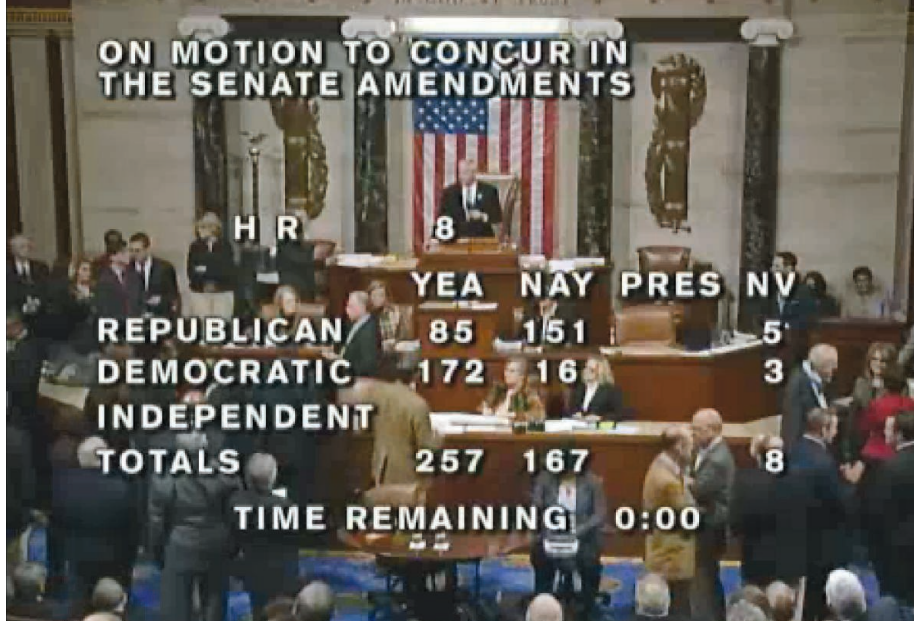
CREDITS: ADAPTED FROM PACALA AND SOCOLOW, *SCIENCE* 305 (13 AUGUST 2004), AND DAVIS, STEVEN ET AL.

New Year's special. Democrats put the tax relief bill over the top in the House vote.

realizing that it contained a mere \$12 billion in savings. (Another \$12 billion in savings is supposed to come from changes in the rules on individual retirement accounts, although many legislators dispute that scorekeeping.) At the same time, reforming entitlements is a nonstarter for most Democrats.

So 18 months after it was crafted as a warning to legislators about the dangers of inaction, sequestration is still bearing down on them like a runaway freight train. And it will take more than lobbying by the scientific community to derail it.

—JEFFREY MERVIS



take seven wedges to simply keep yearly carbon emissions—then 7 billion tons—stable for 50 years. Now, however, annual emissions have risen to 10 billion tons, and the new study estimates that just maintaining that rate

Online

sciencemag.org

For an edited e-mail conversation between Steven Davis and Robert Socolow, go to http://scim.ag/climate_wedge

will require two additional wedges. That makes nine. An additional 12 wedges might be needed, Davis and colleagues estimate, if the global economy doesn't "decarbonize" in the way the original study expected. Economic forecasters back then predicted that economic and technological changes—such as a shift from oil to natural gas—would slowly reduce the amount of carbon emitted per unit of economic output. Instead, the opposite has happened, driven mostly by Chinese and Indian fossil fuel use. As a result, up to 12 "hidden" wedges could be needed "just to stay at what we thought

was business as usual," Davis says.

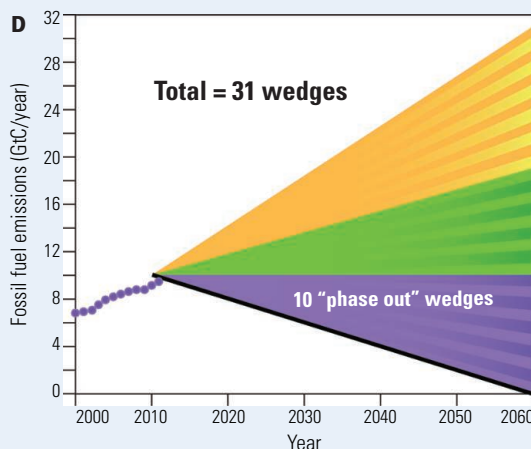
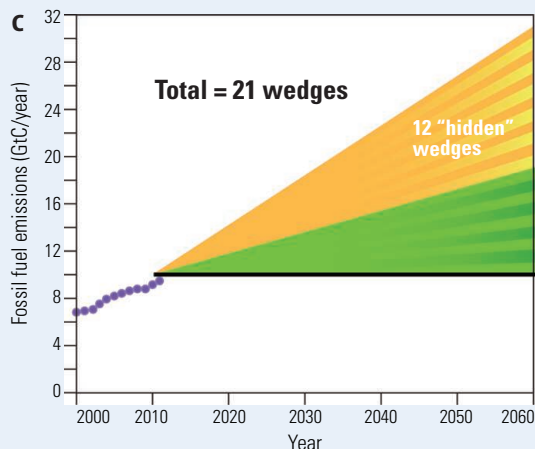
Finally, the authors estimate that an additional 10 wedges will be needed to actually phase out carbon pollution and avoid major temperature increases. The original paper called for maintaining stable annual emissions until 2054 and then subsequently cutting them by 2% annually. But a climate model used by Davis's team suggests that approach would raise global temperatures by 1.52° to 2.32°C above preindustrial levels by midcentury. So "holding emissions constant for 50 years will not prevent us from exceeding 2°," Davis predicts, citing the warming limit that nations agreed upon at the Copenhagen climate summit in 2009. The goal should be zero emissions in 50 years, the authors say. The bottom line: It could take 19 to 31 wedges to reach that goal.

Socolow doesn't dispute the new paper's arithmetic or its daunting scientific conclusions. But he says it's time to move "beyond accounting discussions" to actual policy and action. Eight years since wedges first joined

the climate lexicon, humanity is nowhere near achieving even a single one, he and others emphasize. (Socolow does point to the recent expansion of natural gas use, however, as a rare sign of progress.)

The new calculation also "raises, implicitly, the intriguing question of how to choose among goals," Socolow adds. The original study, he says, focused on "difficult but achievable goals" so as to balance the potential risks of warming against the potential risks posed by some of the solutions. For example, if an expansion of nuclear power is done recklessly, he says, it could proliferate weapons material. The latest update highlights the need for making a careful "balance of investments" in both short- and long-term solutions, says climate specialist Daniel Schrag of Harvard University. "New technologies really are needed," he says. And he and others agree that one simple assertion from the 2004 paper still holds true: "The choice today is between action and delay."

—ELI KINTISCH



(C) The new study concludes that if the economy doesn't decarbonize as forecast, stabilizing emissions may require tackling 12 more "hidden" wedges. (D) It also concludes that 10 additional wedges would be needed to phase-out emissions by 2060.

INFECTIOUS DISEASE

Approval of Novel TB Drug Celebrated—With Restraint

On New Year's Eve, the U.S. Food and Drug Administration (FDA) approved the first new tuberculosis drug in more than 40 years. But the celebration was tempered because sobering challenges face the drug's widescale use.

FDA approved the drug, bedaquiline, for only patients who have multidrug-resistant tuberculosis (MDR TB), which can require up to 2 years of treatment. *Mycobacterium*



Goodwill dividend. Janssen's Koen says the company OK'd bedaquiline R&D despite the lack of a market.

tuberculosis easily develops resistance to drugs used alone, including bedaquiline, so FDA specifies that the pill must be part of a combination therapy. Recognizing the urgent need for better MDR TB drugs, FDA put bedaquiline, made by Janssen Therapeutics of Titusville, New Jersey, through an accelerated approval process, which relaxes efficacy data requirements, and approved it in 6 months. "This is indeed a major breakthrough," says Nesri Padayatchi, a TB specialist at the Centre for the AIDS Programme of Research in South Africa in Durban. But Padayatchi, who works in a country with a high MDR TB burden, immediately adds, "I am skeptical about its use in our setting."

In South Africa and many other countries, MDR patients often start treatment without having the results of drug-sensitivity tests—which are both costly and time-consuming—of their particular *M. tuberculosis* strains. As a result, patients sometimes receive drugs that they are resistant to, and adding bedaquiline would be essentially giving monotherapy—and breeding resistance to it. "Bedaquiline is indeed a great drug, but we have to just be careful about how we use it," Padayatchi says. Clinician Andreas Diacon of Stellenbosch University, Tygerberg, in South Africa, who ran one of the clinical trial sites that tested bedaquiline, says its "major impact" may well be to "kick-start" the routine tailoring of drug regimens to each patient's MDR strain.

Bedaquiline's discovery was first reported in *Science* 8 years ago (14 January 2005, p. 223) by a team led by Koen Andries, who works at Janssen in Beerse, Belgium. The drug raised high hopes because it seemed extraordinarily powerful and worked by a unique mechanism: It inhibits an ATP enzyme that is specific for mycobacteria, which they need to convert energy for reproduction. Even so, Andries says it was a "rough ride" to develop, because there's no attractive marketplace. Wealthy countries have scant tuberculosis and a minuscule number of MDR TB cases—only 98 occurred in the United States in 2011. But he praises his bosses for sticking with it, and says Johnson & Johnson (J&J), which owns Janssen, plans to sell the drug at little profit in developing countries that approve it. (FDA regulates U.S. use.)

In 2011, 12 million people had active cases of TB, according to the World Health Organization (WHO). Relatively safe drugs can cure drug-sensitive TB in 6 months, but an estimated 630,000 people worldwide have the much harder to treat MDR TB. Nearly 1.4 million people died in 2011 from TB because they weren't properly diagnosed, skipped drug doses, received ineffective second-line treatments, or had concomitant infections with HIV. By adding bedaquiline to existing regimens, researchers hope to replace the most toxic drugs, shorten the course of treatment, and cure more MDR TB cases. Ultimately, combinations of bedaquiline and other novel drugs in the pipeline may cure conventional TB cases more quickly.

FDA based its approval of bedaquiline on two studies that involved 440 MDR TB patients. Both added bedaquiline to standard regimens and evaluated how long it took patients' sputum to clear *M. tuberculosis*, a surrogate marker for a cure. One trial found that patients given bedaquiline became sputum negative for the bacilli after 83 days versus 125 days in patients taking a placebo. In a second trial that did not have a placebo arm, sputum became negative after 57 days. Phase III studies in MDR TB patients just getting under way—which may take 5 years to complete—will evaluate whether adding bedaquiline to existing regimens indeed decreases disease and death.

Serious side effects included increased irregular heart rhythms and more deaths in the treated group of the placebo-controlled study (11.4% vs. 2.5%), which Janssen must note in the packaging of the drug. Andries doubts the drug harmed patients, however: Not only did the causes of death differ and have no connection to abnormal heart rhythms, but most occurred a year after people stopped receiving the compound. "It would be very weird and unusual that side effects would appear after 1 year of stopping bedaquiline treatment," Andries says.

TB researcher Barry Bloom of the Harvard School of Public Health in Boston says several logistical questions loom large. Given the WHO estimate that 81% of the people who have MDR TB do not know it, how will suppliers determine how much bedaquiline to procure? How should clinicians best combine it with existing TB drugs? Will bedaquiline interfere with drugs to treat HIV or other diseases? "You have to take this a step at a time," Bloom says.

As for bedaquiline's future as a first-line treatment, J&J gave the Global Alliance for TB Drug Development (TB Alliance), a non-profit based in New York City, a royalty-free license to develop and sell it worldwide for that use. Mel Spigelman, head of the TB Alliance, says tests in a highly predictive mouse model combined bedaquiline with two or three other new TB drugs in development and reduced the 6-month cure time to 6 weeks. "I think there's a huge potential," Spigelman says. They plan to launch human studies later this year.

Delighted as Spigelman is about bedaquiline's approval for MDR TB, he cautions that this is just a first step. "The only downside of getting the approval now is people walk away and say, 'Ah, it's a new drug, the problem is solved,'" he says. "It's not yet. This is the beginning."

—JON COHEN

CHINA

Making a Selfish Generation by Fiat

SHANGHAI, CHINA—Selfish, spoiled, and maladjusted: Stereotypes about only children live large in China, where a generation of singletons has lately reached adulthood. Media commentators regularly bemoan the materialism and egocentricity of those born after the introduction of the one-child policy in 1979. First they were called “little emperors.” Now that they are grown up, *China Daily* has dubbed the demographic the “spoiled generation.”

But until recently, there was little empirical evidence to back up such claims. In a paper published online this week in *Science*, Lisa Cameron of Monash University in Melbourne, Australia, and co-authors attempt to measure the degree to which the one-child policy has shaped the personalities of Chinese 20- and 30-somethings. The economists use games from experimental economics to assess qualities like altruism and competitiveness in cohorts born just before and just after implementation of the one-child policy. After controlling for gender, education, and other factors, those who became only children because of the one-child policy were considerably less trusting, less trustworthy, less conscientious, and more risk-averse than other participants.

Some scholars say the study lays a solid foundation for further investigation into the social effects of the one-child policy. “This paper is a very nice example of the productive use of experimental economics to study questions of broad significance for the social and behavioral sciences,” says Simon Gaechter, an experimental economist at the University of Nottingham in the United Kingdom. Others caution that sorting out the influence of confounding factors in any analysis of the one-child policy is complex and far from complete.

Few regulations have so indelibly transformed a society as the one-child policy. Over the past several decades, scholars have detailed the demographic and economic consequences. Citing such factors as a rapidly aging population and a skewed sex ratio at birth, some Chinese scientists have been pushing their government for years to relax the policy (*Science*, 17 September 2010, p. 1458). But less is known about the social impact, says demographer Wang Feng, director of the Brookings-Tsinghua Center for Public Policy in Beijing. “We are looking at only children in an only-child society,” Wang says. “It’s really hard to get a control group.”



Little emperor. Stereotypes about China’s only-child generation abound, but lack of a control group makes them difficult to test.

To tackle that problem, Cameron and colleagues compared four cohorts living in Beijing who are just a few years apart in age—a total of 421 subjects born in 1975, 1978, 1980, or 1983. The imposition of the one-child policy made a stark difference in family composition. Roughly 27% of the subjects born in 1975 were only children. By 1980, that had increased to 82%.

The researchers had participants play four economics games. In the Trust Game, for example, players allotted \$16 each were paired with an anonymous partner and asked to give some money to their partner, knowing that the amount would be tripled by the experimenter. The partner could then return a portion of the money to the first player—a design meant to reveal how much participants trust others and can be trusted in return. Using an instrumental variable approach, a technique employed in economics to control for selection bias, the authors identified participants who were only children as a result of the policy. These subjects gave on average 16% less money

to their partner than other participants gave. They also returned 11% less of what they received than did their counterparts.

The study is “groundbreaking in its empirical documentation of the relationship between historical change and social psychology,” says Vanessa Fong, an anthropologist at Amherst College in Massachusetts who examined social effects of the one-child policy in the 2004 ethnography *Only Hope: Coming of Age under China’s One-Child Policy*. Fong surveyed more than 2000 singletons about their attitudes and habits, arriving at similar conclusions about a number of traits. But, she adds, attributing differences among birth cohorts to the one-child policy alone is dicey. “The behavioral propensity of a generation depends on a lot of factors beyond the family level,” agrees Gu Baochang, a demographer at Renmin University of China in Beijing. He notes that the one-child policy was unveiled during a “period of dramatic societal transformation.”

Other scholars assert that age differences may have skewed subjects’ decisions in the economics games. “Teasing out age, period, and cohort is a classic identification problem,” says Yong Cai, a demographer at the University of North Carolina, Chapel Hill. Cameron says her team included the 1975 and 1983 cohorts in part to identify peer effects, or the impact of growing up surrounded by other only children. That did not turn out to be a significant factor, and when the researchers excluded these two cohorts from their analysis, they got results similar to their overall findings—suggesting that age had little effect.

Perhaps the biggest surprise of the study is how thoroughly the only-child subjects lived up to their bad reputation. Psychology studies had largely found “no difference between only children and those with siblings,” Cameron says. Most of those studies, however, were done in the West and involved parents who presumably chose to have one child. By eliminating the bias of parental choice, the one-child policy is what Cameron calls a “natural experiment” for studying sibling relationships.

Others would like to see similar studies of only children in countries like Japan in which rapidly declining fertility is not by government directive. Another open question is the one-child policy’s effect on younger cohorts, including the children of China’s “little emperors.” Those youngsters lack not only siblings but also cousins, aunts, and uncles. “If anything,” Cameron says, that should “magnify the effect.”

—MARA HVISTENDAHL

IMAGE MANIPULATION

Author of Popular Blog That Charged Fraud Unmasked

She called herself the “fraudster,” and offered up these biographical details: She was a tenured female scientist with “over 100 peer reviewed papers in the biomedical literature.” And she was fed up with current systems to address scientific misconduct. Last summer, she launched www.science-fraud.org, an acerbic blog dedicated to unmasking such wrongdoing, which quickly gained thousands of fans. Thanks largely to tips from readers, the blog accused dozens of often prominent scientists of image manipulation in published papers, backing up claims with detailed analysis of figures.

Last week, the fraudster’s cover was blown and the site shut down. She turned out to be a he—Paul Brookes, a 40-year-old, highly respected mitochondrial biologist at the University of Rochester Medical Center in New York, who originally hails from the United Kingdom. The details of the unmasking remain murky. An accuser identified Brookes via e-mail to dozens of scientists and university administrators, including the president of Brookes’s own university. In the e-mail, obtained by *Science*, the author, who appeared to have used an anonymous Gmail address, described [science-fraud.org](http://www.science-fraud.org) as a “hate website” and urged those named on it to file “an official legal suit.”

Brookes confirmed on his personal blog that he was, indeed, the fraudster. He removed all content from [science-fraud.org](http://www.science-fraud.org). He expressed a mix of defiance—“apparently anonymity is OK if you’re inciting lawsuits but not if you’re investigating scientific misconduct,” Brookes wrote on [science-fraud.org](http://www.science-fraud.org)—and contrition. In an interview with *Science*, he admitted that his lumping all suspicious images under the rubric of fraud may have been a tactical error. He hunkered down, hoping Rochester wouldn’t fire him and that he wouldn’t be flooded with lawsuits. There are “100 plus people who can file suit against me,” he told *Science*. None of those accused of misconduct on [science-fraud.org](http://www.science-fraud.org) whom *Science* contacted would comment.

Brookes’s employer was not pleased to learn of his blog. “While we respect his right to free speech, we cannot condone the manner in which he raised these critically important questions,” wrote Teri D’Agostino, a spokesperson for the medical center, in an e-mail.

“We will immediately develop a plan with Dr. Brookes to ensure that he follows our policies for any University-related activity and that his personal activities continue to be kept entirely separate from his University role.”

Many of Brookes’s colleagues, who were unaware that Brookes was the anonymous critic, agree that although the blog may have gone too far, it tapped into a real problem—and that from what they knew of his character, Brookes had probably started it out of genuine

posted the case online for all to see.

By Brookes’s count, [science-fraud.org](http://www.science-fraud.org) posted about 100 accusations. More than a dozen of those papers have been corrected or retracted. Still, Brookes may have opened himself up to charges of defamation with his presumptions that the scientists he named had committed fraud. “You’ve got someone who is publishing on a blog statements that are allegations of fact,” meaning they are “provably true or provably false,” says Katharine Larsen,

an attorney at Levine Sullivan Koch & Schulz in Philadelphia, Pennsylvania, whose focus is defamation and free speech. Furthermore, she says, although “there is a constitutional right in the first amendment to speak anonymously,” that’s balanced against other rights, such as the right of an aggrieved party to seek redress for an alleged injury.

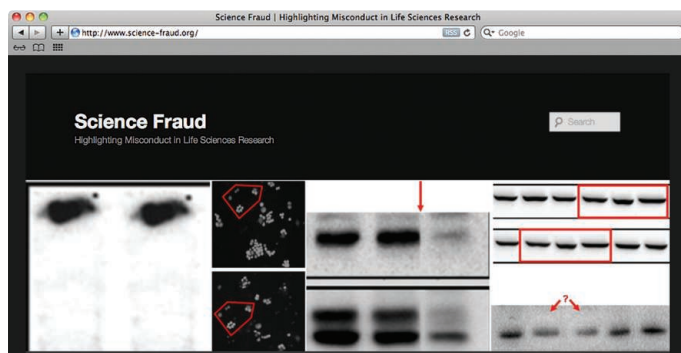
There’s no question that image modification is widespread—but gauging the intent behind it isn’t always easy. *The Journal of Cell Biology* (*JCB*) has performed

image analysis prepublication for just over a decade. One percent of papers have their acceptance revoked for inappropriate image manipulation. Another 25%, however, have at least one figure that’s been modified in violation of *JCB*’s rules—for example, by exaggerating contrast—but in a way that doesn’t alter interpretation of the results. In those cases, the journal asks the authors to remake the figure or figures with a more accurate representation of the original data and then publishes the paper, says Mike Rossner, executive director of Rockefeller University Press, which publishes *JCB*.

While Brookes waits to see whether any lawsuits come his way, he’s hoping to continue his efforts under his own name. He would still welcome anonymous tips. “Just because this site went away, the need for an anonymous postpublication peer review process is still there,” he says.

Mike Murphy, a mitochondrial biologist at the Medical Research Council in Cambridge, U.K., and a longtime collaborator, agrees. “It would be a terrible pity if a legal hammer was used to smash” Brookes’s efforts, he says. “We can’t just say science is self-correcting. There’s a problem, and we really need to face up to it.”

—JENNIFER COUZIN-FRANKEL



Risky business. Biologist Paul Brookes started his site, www.science-fraud.org, out of frustration with publication integrity.

concern and frustration. “This is not a person doing this because of anger and sour grapes,” says Victor Darley-Usmar, a biochemist at the University of Alabama, Birmingham, who was Brookes’s postdoctoral adviser. “He does very, very thorough work, very careful work,” Darley-Usmar continues. “I’ve been proud to be associated with him.”

Brookes says the impetus for [science-fraud.org](http://www.science-fraud.org) came from his own failed efforts to correct what he saw as discrepancies in published literature. Journals he contacted requested that he be named in accusations to the original authors. “That requires me to go on the record in my field accusing one of my peers of misconduct,” he says. He considered that option a nonstarter.

With [science-fraud.org](http://www.science-fraud.org), Brookes hoped to “speed up the process of resolution.” He says he received tips from between 50 and 100 anonymous e-mail addresses, though a core group of about six people contacted him regularly. The tips generally listed the name of the paper and a description of the purported problem. Brookes then pulled the original paper from his university’s library. Sometimes, he says, “I couldn’t see what the person was talking about and I just wrote back politely and said, ‘Thanks, I don’t see a problem.’” But in other cases, he sided with the tipster and



Up, up we go. This family in Queens, New York City, is part of the study's elaborate pilot phase.

The Children's Study: Unmet Promises

Having spent about \$1 billion over the past decade, a U.S. study of environmental influences on children's health is still trying to define its methods. Critics say it has gone astray; advocates say it is on track at last

FOR EPIDEMIOLOGIST MAUREEN DURKIN, the end of her dream project came in June, when a moving van pulled up in front of rented office space in the western suburbs of Milwaukee. There, her staff of 15 based at the University of Wisconsin, Madison, had spent 7 years and \$16 million in federal dollars gearing up to recruit 1250 pregnant women in Waukesha County whose babies would be among 100,000 followed for the largest ever long-term study of U.S. children's health. Instead, after a pilot phase in which Durkin's group enrolled 200 women, the National Institutes of Health (NIH) told Durkin that a private contractor would be taking over her part of the study. Over 2 hot summer days, her staff members wheeled freezers and centrifuges, computers, and blood tubes into the van for recycling.

"We spent millions training and getting the equipment and getting ready to go," Durkin laments. Now, she says, NIH is "just throwing it down the drain."

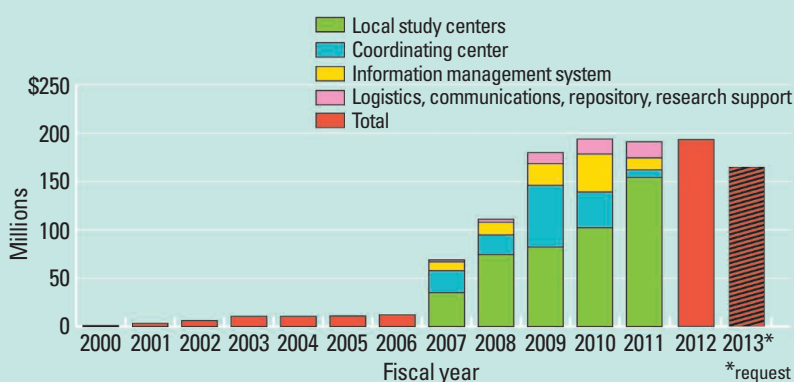
The closure of Durkin's study site and 39 others like it reflects an abrupt shift in course for one of the most ambitious U.S. biomedical research projects ever: the National Children's Study (NCS). Twelve years ago, Congress

hasn't said how it will achieve that.

Onlookers wonder how a seemingly simple epidemiology project—enrolling pregnant women for a long-term observational study—became hopelessly complicated and costly. NCS became "weighted down" with add-ons like "a ship encrusted with barnacles," says pediatrician and epidemiologist Nigel Paneth of Michigan State University in East Lansing. He was an investigator in the original NCS but was dropped, and hasn't yet been invited to join the new one. After investing \$1 billion over 12 years, NCS has produced little, critics say, even as similar studies in other countries have surged ahead.

"I find that so depressing. The missed opportunities, how much could have been accomplished," says Brown University epidemiologist David Savitz, an

Funding for U.S. National Children's Study



Billion-dollar test run. Funding for NIH's children's study has totaled \$992 million through 2012, including \$114 million for a contract for a coordinating center that was discontinued.

CREDITS (TOP TO BOTTOM): SUZANNE DECHILLO/THE NEW YORK TIMES/REDUX; NATIONAL CHILDREN'S STUDY

October 2000 Children's Health Act calls for NICHD to lead long-term child development study

March 2004 NCS planners decide to recruit 100,000 pregnant women from random sample of U.S. households in about 100 counties. Costs estimated at \$2.7 billion over 25 years



September 2005 Contracts awarded for first six "vanguard centers" and data coordinating center

February 2007 Despite opposition from NIH Director Elias Zerhouni, Congress appropriates \$69 million to launch study; number of funded study centers rises later in year



May 2008 Institute of Medicine endorses household sampling, but says it must be pilot tested

January 2009 New Obama administration backs study, recruitment begins at seven vanguard sites

March 2009 NIH acting director Raynard Kington tells Senate panel that "true costs" are higher than expected—up to \$6.9 billion



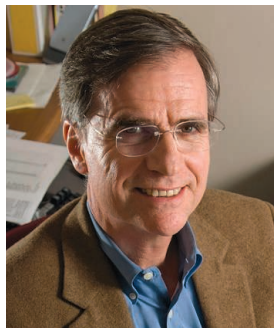
early adviser to the project. Others go further: NCS has been "a national embarrassment. It is a disaster," says a prominent U.S. epidemiologist who asked not to be named. Former NIH staff members and investigators blame the problems on a confluence of factors: a mandate forced on NIH by Congress, a lack of strong scientific leadership, funding uncertainties, and a plan that embraced too many objectives. Along with bureaucratic hurdles, this led to delays and missteps.

NIH says it has now figured out how to run a scientifically robust children's health study with a smaller budget. But some of those who envisioned a 20-year survey of children's health now have doubts about whether it can be pulled off. The project's implosion, Paneth says, "is quite an amazing chapter in the history of American science."

The perfect study

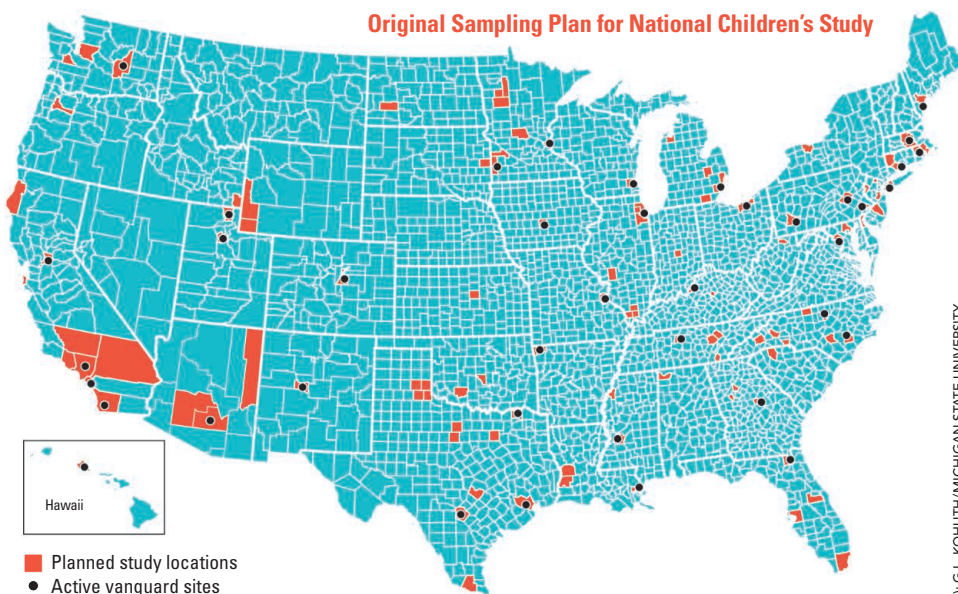
The idea for NCS arose in the 1990s, during the Clinton administration, amid growing concerns that chemicals in the environment might contribute to childhood diseases such as autism and cancer. The inspiration came in part from an influential 1993 National Research Council report led by Philip Landrigan, a pediatrician at Mount Sinai School of Medicine in New York who had done studies linking low-level lead exposure and lower IQ. The report found that current safety levels for chemicals, based on adult data, might not protect children.

A White House task force on children's health proposed a long-term study to look for links between health, development, and environmental influences—defined broadly to range from chemical exposures to psychosocial factors. In 2000, Congress passed a law directing NICHD to lead the study in cooperation with the Environmental Protection Agency (EPA) and others.



The study's problems are "an amazing chapter in the history of American science."

—NIGEL PANETH,
MICHIGAN STATE UNIVERSITY



Diverse picture. To represent the U.S. population, the National Children's Study set out to recruit pregnant women living at specific addresses within a random sample of 105 locations, mostly single counties.

Although some researchers worried that a large study would siphon funds from individual-investigator research grants, others saw it as a successor to the Collaborative Perinatal Project (CPP)—a landmark NIH-funded study of 55,000 children in the 1960s and early 1970s. Among key results, it found that cerebral palsy usually develops long before delivery and that infections are a factor in pregnancy complications.

Epidemiologist Mark Klebanoff of Ohio State University College of Medicine in Columbus recalls an NCS planning meeting in late 2000 led by then-U.S. Department of Health and Human Services Secretary Donna Shalala with six experts on long-term health studies. "Everybody thought it was a great idea." Says Klebanoff, then at NICHD: "The advice we got was to be bold." Following the lead of a Danish children's health study already under way and another starting in Norway,

planners chose a size of 100,000 children, large enough to study rarer diseases such as type 1 diabetes. An early cost estimate was \$2.7 billion, spread over 25 years.

A fundamental problem was that the new administration in 2001, headed by George W. Bush, didn't like the project because of its cost and didn't include it in its 2002 funding request. So with a mandate from Congress and a small budget scraped together from several agencies, NICHD formed a study office in 2002 headed by pediatrician Peter Scheidt.

Heeding Congress's request for broad input, Scheidt oversaw the formation of dozens of working groups and advisory committees involving more than 2500 experts. They came up with 28 overarching hypotheses exploring, for instance, a potential link between violent video games and gun injuries, and whether genetics make some children more vulnerable to harm from pesticides. Researchers would collect numerous samples, such as placentas and tap water in homes, and follow children through medical exams, home visits, and phone interviews with parents.

The study "became the vehicle for most hypotheses related to children's health," says

July 2009 Pilot data indicate that recruitment by knocking on doors could take 9 years, 5 more than planned

July 2009 Scheidt removed as NCS director

September 2009 Duane Alexander steps down as director of NICHD. Alan Guttmacher later becomes director



October 2009 Acting NCS director Steven Hirschfeld announces study will focus on “data collection,” not specific hypotheses



Spring 2012 New contractors begin to take over all 40 academic study sites

July 2012 New study proposal would recruit half of women at birth and half during pregnancy by sampling U.S. hospitals, not by door-to-door surveys

January 2013 IOM workshop to review new study plan



2014 Revised children's study to begin recruitment

Jonathan Samet, an epidemiologist at the University of Southern California in Los Angeles and an adviser at the time. But that was a problem, says epidemiologist Lynn Goldman of George Washington University in Washington, D.C., who, as an EPA official in the 1990s, helped conceive the project: “It was a tabula rasa. Whatever you wanted it to be, it would be that.”

Perfectionism was a drag on the study, critics say: Scientists debated at length how best to recruit pregnant women. CPP had found them through university clinics. But NCS's social scientists successfully argued for a novel strategy: Recruiters would contact or knock on doors of randomly chosen households, much as the census surveyors do. This would ensure a broad sample and reach underrepresented groups such as the uninsured. But the strategy was untested.

Federal statisticians determined the sampling frame—a randomly chosen set of 105 sites, mostly single counties, from rural Minnesota to Queens, New York City. In September 2005, with a still-modest \$11 million annual budget, NICHD funded its first six vanguard centers to begin recruitment. These were to be activated once Congress approved funding for the study.

All systems go

Then in 2007, NCS's fortunes changed. Backers had won champions in congressional districts representing some of the 105 counties to be sampled. That year, lawmakers approved \$69 million in startup money. With that in hand, NCS leaders announced another 22 centers, saying they would recruit women in waves, and inked a contract with Westat, a private contractor, to centralize data collection.

A crucial element was missing, however: The study had not yet gone through outside peer review. NIH sent its 700-page research plan to the Institute of Medicine (IOM) in June 2007 for a fast-track review. Eleven months later, an IOM panel endorsed the household sampling plan. But reviewers also warned that NIH needed to test this approach in a pilot before launching the study. Scheidt's office responded that they would add more time to the vanguard

recruitment phase, delaying the full study.

The now-seven vanguard sites began recruiting the first pregnant women early in 2009—2 years later than originally planned. The new administration under Barack Obama supported NCS. But when acting NIH director Raynard Kington asked for updated budget projections, NCS staff members told him the full cost estimate had grown to as high as \$6.9 billion, more than twice the original estimate. Kington was outraged, according to a former NCS staffer—and so were Senate staffers. At a Senate spending panel hearing in March, Kington said his staff was working to “assess true costs” and make “adjustments” to the study. In an unusual reprimand, the panel accused NIH in a report of withholding information and “a serious breach of trust.”

NICHD Director Duane Alexander explained that early budget projections were “rough and conceptual” and did not include overhead or inflation, according to a summary of his statement. Behind the scenes, Scheidt's staff members protested that they had followed instructions from the NIH budget office not to update a 2005 cost estimate of \$3.1 billion because the White House planned to ask Congress to zero out funding for NCS. In July 2009, the president announced his choice for the new NIH director: Francis Collins, the former chief of NIH's genome institute. That month, Scheidt was removed as NCS director, and 2 months later, Alexander stepped down as NICHD chief to take another position at NIH.

Course correction

Steven Hirschfeld, NICHD's clinical director and an expert on childhood cancer, took over as acting chief of NCS. Hirschfeld announced major changes. “Everything got turned upside down,” says Goldman, who headed a study site until 2010. If Scheidt gathered too much

advice, under the new director the pendulum swung the other way, according to NCS investigators, who say Hirschfeld made decisions internally without consulting them. For example, pilot research at the vanguard centers would be used to work out logistics and costs of data collection, not to recruit the first women for the study.

To the consternation of many, Hirschfeld also announced that NCS would no longer be a hypothesis-driven study, but a “data collection” platform for exploring hypotheses that would be added later. Hirschfeld's office dissolved protocol committees.

Investigators worried that this might yield data that wouldn't be very useful. “How do you characterize a group of women in the absence of hypotheses? What questions do you ask them, what phenotypic measurements do you collect?” asks epidemiologist Roberta Ness of the University of Texas Health Science Center, Houston.

Meanwhile, as the data came in from the seven vanguard centers, it became clear that it would take 9 years—twice as long as originally thought—to enroll 100,000 pregnant women through contacting households. Some investigators weren't surprised. Because pregnancy is a brief period in a woman's life, Goldman says, “to

find women right at that time is not easy.” (Others say the strategy didn't get a fair test; it worked “fabulously” at some sites, Durkin says.) Deeming household recruitment a failure, the NCS office decided to designate 30 more study sites as vanguard centers and use all of them to test other recruitment strategies, including recruiting women at doctors' offices.

But what seemed like a logical plan became a bureaucratic nightmare, study site investigators say. Any significant change to a protocol, such as a reworded questionnaire, had to be cleared through the Office of Man-



“It's hard to argue that our 10 years of planning has been more valuable than the 10 years of progress made by others ...”

—DAVID SAVITZ,
BROWN UNIVERSITY

agement and Budget. Another complication was that Hirschfeld allowed the Westat contract for a central database to expire, after \$114 million had been spent on it, because it was not “cost effective.” Instead, researchers were told to develop their own data systems, but NCS kept changing the specifications.

Because of the delays, staff members were hired but had nothing to do for months. Then as the centers got up to speed and began enrolling pregnant women, NCS told investigators to stop in November 2011 so that the program office could evaluate the data.

As the pilot phase of NCS “lurched” along, as one researcher puts it, some observers sounded an alarm. Savitz and Ness, who both had run vanguard sites for a time, co-authored a lengthy commentary in September 2010 in the journal *Epidemiology* warning that the study was “still in trouble” and needed “fundamental” changes to improve efficiency, including a prompt switch from knocking on doors to recruiting subjects through doctors’ offices.

The study seemed to veer in an entirely new direction last February. NIH announced that none of the recruitment strategies being tested were feasible. Instead, NIH said it planned to abandon the 105 county sampling plan and switch to a “convenience sample” from health maintenance groups, in order to cut annual costs by 15%, to \$165 million in 2013. A total of 40 study site contracts would not be renewed, and the women they had recruited for the vanguard study would be followed by new contractors.

Two of NIH’s advisers on NCS blasted the agency over the sudden change of course and quit. Congressional overseers also found fault. NIH has since moved toward a compromise: Women will be recruited from a random sample of U.S. hospitals and prenatal care providers affiliated with the hospitals. To save money, perhaps half will be identified when their baby is born, and half when they seek prenatal care.

Righting the ship?

Now that they’ve settled on an efficient study design, NIH officials say, the way forward is clear. The goal set by Congress in 2000 hasn’t changed, Hirschfeld says: The study will still examine the effects of environmental and biological influences on U.S. children’s health and development from before birth until age 21. Gutmacher says the study’s pilot stage “is performing as intended, providing needed hard data on which to base the science and logistics of the much larger main study.” The \$1 billion spent so far was worth it, adds NIH Director Collins: “You



The study’s startup phase “is performing as intended, providing needed hard data on which to base the science and logistics of the much larger main study.”

—ALAN GUTMACHER,
NICHD

would not want to undertake this without being really sure that the model was going to work.” Hirschfeld says that the study has also developed “innovative approaches” and “tools” that will be used “by scientists around the world.”

NIH plans to gather more advice on the study design at an IOM workshop scheduled this week and at a meeting in the spring. Recruitment will likely begin in 2014, Hirschfeld says.

Some former NCS study site investigators support the new strategy. Running the study through 40 contracts with academic centers became “enormously inefficient,” says pediatrician Neal Halfon of the University of California, Los Angeles, who ran one of the largest sites. Halfon and Jørn Olsen, director of the Danish National Birth Cohort, say it makes sense to define specific hypotheses later. “Research is moving forward so fast, most of the hypotheses would be outdated” if defined now, Olsen says. Like some others, including Savitz, Olsen feels it’s not essential that NCS recruit a representative sample of U.S. women.

Advocates of the original NCS vision, however, worry that the goals may be lost. The project’s credibility is already waning in communities where the academic sites are being terminated, they say. If NIH’s plan for sampling hospitals does not recruit a representative sample of U.S. women, it may miss important groups. Recruiting only 50,000 of the women during pregnancy will reduce the statistical power to detect the effects of developmental influences in the womb, others add. Landrigan says he is “very much concerned” that the new, cheaper design “has much lower likelihood ... of discover-

ing potentially causal associations between toxic chemicals and disease in children.”

Most of the academic NCS study site leaders, including Paneth, have signed a white paper arguing that the study must return closer to the original 105 county plan. NCS is spending more than \$3 million a week, points out Michael Bracken of Yale University, a former NCS researcher—money that could go to investigator-initiated grants. In his opinion: “This thing has either got to be done properly or it should not be done at all.”

Who is to blame for NCS’s problems? Congress tops the list, some observers say. Mandating the study without buy-in from top NIH leadership was a recipe for disaster, says a former congressional staffer. But the NIH director played a role, too: Once it became clear that Congress was going to fund it despite NIH’s objections, “this project was so big and so important that it should have been watched more closely,” says another Washington policymaker who asked not to be named.

Scientists are to blame as well by designing an overly complex study, many of those involved with NCS say. “It tried to do too much. But if it hadn’t, it might have never gotten started. It’s a bit of a catch-22,” Klebanoff says. “You could say it was born with such ambitions that the grand vision may not have been realizable,” Samet says.

If the redesigned NCS is to succeed, Savitz says, it needs “an experienced, respected, authoritative, politically astute individual to pull all this together.” The white paper authors say that this person should be an epidemiologist who has run large cohort studies. But Savitz admits he has “little confidence” that such a person would take command.

During the decade when U.S. leaders discussed how to recruit pregnant women for NCS, the Danish and Norway children’s studies, which recruited for less than \$50 million each, have followed children for up to 16 years and have published scores of papers. These studies collected relatively little data on exposures and were able to keep costs down by drawing on national health care records.

At least a dozen large birth cohort studies are now under way in other countries, Savitz notes. “It’s hard to argue that our 10 years of planning has been more valuable than the 10 years of progress made by others with far less money. We’ve missed the boat and need to have a good look at what is going on to see where we can contribute.”

—JOCELYN KAISER

CONSULTING

Expert Firms Play a Hidden Role In Connecting Science and Finance

What every academic and clinical researcher should know before signing up with an expert network firm and interacting with its clients

On 20 November, the U.S. government unveiled what it called the largest insider-trading scheme in its history. According to documents in the U.S. district court in New York, a hedge fund analyst, Mathew Martoma, generated \$276 million in ill-gotten gains for his company, CR Intrinsic Investors, through stock trades based on confidential information about the results of a clinical trial for a new Alzheimer's drug. Prosecutors say that Martoma's inside knowledge of how the drug performed came from a prominent neurologist at the University of Michigan, Sidney Gilman, in a relationship arranged by a third party.

That third party was the Gerson Lehrman Group (GLG), the dominant company in a cottage industry that it helped to invent. Martoma's company was a client of GLG, and Gilman was a member of GLG's expert network. The two spoke 42 times between 2006 and 2008.

The case is the latest in a string of high-profile prosecutions that have focused attention on this little-known world of so-called expert network firms. Such firms connect the savviest people in a field with clients willing to pay for the expert's knowledge. Clients, be they financial analysts, venture capitalists, professional consulting firms, or lawyers, submit a request, and GLG sifts through its files to find the right expert in the relevant area. (GLG's networks cover health care, energy, telecommunications, manufacturing, real estate, and a dozen other sectors.) Experts select which offers from GLG to accept, and after the conversation they bill GLG for their time.

But such expert networks are controversial. Financial managers handling billion-dollar stock portfolios are on the prowl for something their competitors don't know, and some clients may cross the line by prodding an expert to break the law and disclose confidential information.

To find out how such expert network firms operate, and why a researcher would participate, *Science* interviewed several of GLG's top-rated experts in health care.

None wanted to be named, for reasons that include a desire to avoid being connected in some way to the Martoma case. (Martoma was indicted last month. The 80-year-old Gilman, who retired from Michigan after charges were filed against Martoma, has agreed to cooperate with authorities and has not been charged.) So their stories, which are real, illustrate some of the tough issues facing researchers who consult through these firms.

Making a match

Alexander Saint-Amand, the president and CEO of the New York City-based GLG, had just graduated from college and was weighing his career options when he met with a doctor who was treating his mother for early-onset, rapidly progressing dementia. Although the biotech sector was awash in cash, the doctor complained that promising treatments for a host of diseases were languishing while venture capitalists poured money into approaches that any practicing physician could have told them were bad bets. Recalling the conversation, Saint-Amand says he thought: "There must be a better way to get this conversation going between the doctors who know what works and the investors who want to fund it."

The first version of GLG, launched in 1998, was an online service that allowed experts to connect with those willing to pay for their expertise. "But the best scientists didn't want to work with just anyone who finds them online," Saint-Amand says. "They wanted to talk with the most sophisticated people in their field, whether in industry or investors." That led to the current system, in which GLG constructs both sides of the network, arranges all the interactions between clients and experts, and manages the paperwork.

"We have not created a market for consulting," Saint-Amand says. "But we like to say that we have professionalized the process." Part of that service, Saint-Amand

says, is to educate and repeatedly remind its experts about federal laws prohibiting the disclosure of proprietary or material information about a company or its products.

Michigan's vice president for research, Stephen Forrest, says the university definitely wants its researchers to "engage" with industry. Those ties are an essential piece of Michigan's attempt to commercialize discoveries by its faculty members, who in 2011 carried out more research than any other U.S. public university, topping \$1 billion. Potential financial conflicts of interest stemming from such relationships, he adds, need to be managed, not prohibited. However, Forrest concedes that Michigan's current rules, which emphasize the disclosure of possible conflicts, would not stop a fac-

"We have not created a market for consulting. But we like to say that we have professionalized the process."

—ALEXANDER SAINT-AMAND,
THE GERSON LEHRMAN GROUP

ulty member who decides to break the law.

In contrast, one of the country's most prominent medical centers, the Cleveland Clinic in Ohio, believes that consulting for financial investment firms like GLG is so fraught with risk that it strongly discourages its physicians from doing so. "We have carved out expert networks from other consulting activities," says Guy Chisolm, a cell biologist who directs the clinic's Innovation Management and Conflict of Interest Program. "We're very skeptical of this activity."

Chisolm worries that "an expert might know things that could cross the line, or that such firms could use our name to attract business and promote their interests." Fewer than 20 of the clinic's nearly 3000 physician-scientists currently engage in the practice, says Chisolm, who also chairs the Forum on Conflict of Interest in Academe for the Association of American Medical Colleges. That number is dropping, he adds, a trend that pleases him.

"My colleagues have said there's an altruistic reason to do it: 'If you know what treatment would help your patients and you see an opportunity to steer investments in

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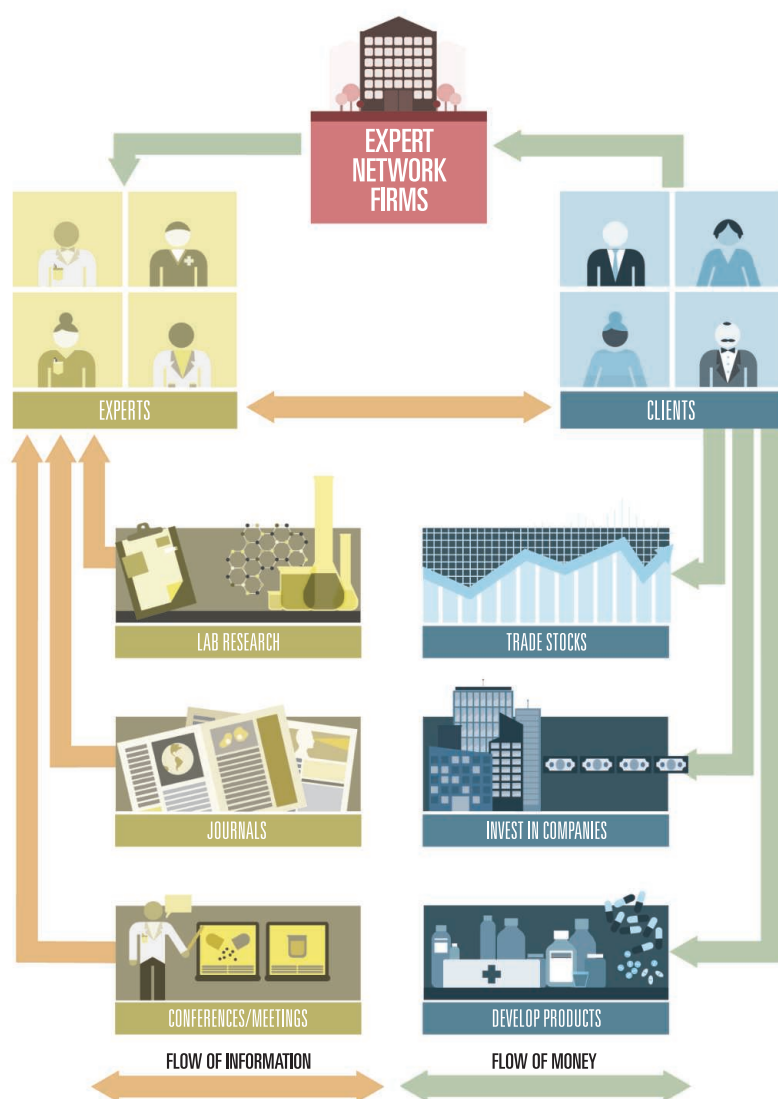
Podcast interview
with author Jeffrey
Mervis (http://scim.ag/pod_6116).

that direction, who better than a Cleveland Clinic doctor to play that role?" But I think the risks outweigh the benefits."

The personal interaction is one of the reasons that Dr. X, a pulmonary specialist at a prominent medical center in the mid-Atlantic region, signed up with GLG. Some firms feed their experts a steady diet

ized in court filings as a "personal" relationship, with Gilman "eventually coming to view Martoma as a friend and pupil." But that description doesn't square with what Dr. Y, a research oncologist at one of the top medical schools in the United States, has experienced as a member of GLG's expert network.

HOW MATCHMAKING WORKS



A complex network. A firm's stable of experts obtain their knowledge from many sources, and its clients put what they've learned to use in many ways.

of surveys, says Dr. X, who also consults with MEDACorp, a rival expert network firm owned by Leerink Swann, a Boston investment bank (see sidebar, p. 139). "But GLG seems to like the idea of putting a warm body with another warm body."

That human connection may have made it easier for Gilman and Martoma to build what federal prosecutors have character-

"It's rare to talk to the same person twice," Dr. Y says, "and I've never talked to anyone more than three or four times. Also, the idea that this is a mentor/mentee relationship is just silly. Mentors don't give their students the answer. Rather, they teach the student to think for themselves."

In fact, the GLG experts who spoke with *Science* say that they don't spend much time

thinking about the person on the other end of the line. "I don't really care who they are," says Dr. Z, a cardiovascular researcher at a major Midwestern medical center. "I can figure out pretty quickly how much they know based on their questions. But my job is simply to provide scientific answers."

Talk isn't cheap

Why do these physician-scientists sign up with GLG? Being an expert, they say, forces them to stay current in the field, and also builds their reputation. Most also see consulting for GLG as an extension of their role as an educator. And some simply enjoy talking about their work with an attentive listener.

But first and foremost, they say, is the money. The three experts quoted in this article have been designated as "leaders," meaning that GLG ranks them in the top 5% of its network. Saint-Amand estimates that leaders log anywhere from 100 to 150 calls a year. And he says the average hourly rate for physician-scientists is \$250.

Dr. Y, the oncologist, says he turned his back on the chance to become wealthy when he chose to go into academic medicine. But he believes that society "undervalues" what he and his research colleagues do for a living. Consulting for GLG doesn't balance the scale, he says, but it's still worth doing.

"I might as well put my medical education to work," he says. "In a good year I clear \$12,000," he says. "That's not a lot, but it's better than nothing."

Consulting also addresses a larger trend in academic medicine, says Dr. Z, the research cardiologist. "Scientists are becoming an endangered species," he says. "We are losing them to clinical care. And the reason is obvious: A classmate of mine [in clinical care] can make \$1.3 million a year, while the starting academic salary is \$150,000."

At the same time, all of the experts who spoke with *Science* say that they would withdraw from the network if they felt that their participation distracted them from doing their academic job. "If I wanted to do more, I'd become a full-time consultant," Dr. Y says. "I'd never put doing this over my research."

Dr. Z says that he's actually trying to cut back. "I don't want this to interfere with my research and caring for patients. I also get e-mails from another expert network firm, but I don't respond to them."

How much time experts spend on consulting is important to their institutions, too. Many research universities and medical schools allow faculty members to consult the equivalent of one day a week. And although

An Expert's Words Can Move Markets

When scientists reported at a 2005 meeting that an experimental drug for stroke victims had improved short-term functioning and didn't increase bleeding, investors in the small biotech company that had developed the drug expected to see a jump in its stock price. Instead, the stock price of Renovis fell by 23%, shedding almost \$100 million in value in less than a week.

What happened? On the first working day after the meeting, neurologist Marc Fisher of the University of Massachusetts Memorial Medical Center in Worcester spoke to a bevy of stock analysts assembled by MEDACorp, an expert network firm owned by the Boston investment bank Leerink Swann. As one of MEDACorp's experts, Fisher raised questions about how the patients' status had been assessed 3 months after the drug, Cerovive, was administered. Within hours Renovis's stock price started dropping, and it never recovered. A year later, Cerovive flunked a second, larger trial, and in 2008 Renovis went out of business.

The former CEO of Renovis, Corey Goodman, believes that Leerink Swann analysts were expecting disappointing results from the drug trial and saw a chance to make money by betting that Renovis's stock price

Renovis

would fall. A rise would have meant a big loss for the investment firm,

so Goodman surmises that it found an expert who hadn't seen the results but was willing to criticize the study anyway. Goodman first raised those suspicions at a 2006 conference of biotech executives, and he repeated them last month to *Science*.

Fisher says that Goodman is wrong. "I attended the presentation [at the European Stroke Conference in Bologna, Italy] and I knew the results," he says. Fisher admits, however, that he didn't initially understand the study's unorthodox use of the Rankin scale used to measure outcomes and that a few days later he modified his criticism. "But the results from the next trial were totally negative, so I guess my initial concerns were valid," he adds.

Fisher stopped consulting for MEDACorp and for drug companies in 2010 when he became editor of *Stroke*, an American Heart Association journal that prohibits its editor from engaging in such activities. "I have no conflicts now because I'm not allowed to do it," he says.

But Fisher agrees with Goodman on one point: "I had no idea my words would have such an impact."

—J.D.M.

most institutions consider consulting for an expert network firm to be part of the broader category of outside activities that must be disclosed by any faculty member with federal funding, there are certain aspects of that relationship that could raise a red flag.

"We do lay out a variety of conditions that need to be followed, including their obligations as a professor, the prohibition against disclosing insider information, protection of intellectual property, and the use of Hopkins' name," says Julie Gottlieb, associate dean for policy coordination for Johns Hopkins University School of Medicine in Baltimore, Maryland. "The amount of money earned could be a trigger to look deeper into the relationship."

At the same time, Gottlieb says, "faculty consult for a range of entities, and there are risks associated with all of those relationships for which they need to take precautions. Being part of an expert network represents something of a gray zone."

On a short leash

The Cleveland Clinic has decided to make doctors who want to enter that gray zone jump through additional hoops. The clinic's law department must first approve all contracts, no matter how much the expected payment. (There's a \$10,000 minimum for other consulting.) After the contract passes muster, the physician who signs up with GLG or another firm gets a black-bordered document with 11 bullet points describing practices that must be followed.

The issue is not consulting per se, Chisolm says, but rather who's listening to the doctor's advice. "When you're talking to Merck or Medtronic, your expertise is going to be used to help the company make decisions on the direction of its research, or improvements to its products," he says. "You know more about the recipient than you do when you're involved with an expert network." The opportunity for insider trading exists in both cases, Chisolm observes, "but



—GUY CHISOLM,
THE CLEVELAND CLINIC

the chances are much higher when you're talking to investors."

Saint-Amand says the firm's compliance practices are designed to eliminate that risk. "We provide this framework with audit trails. And we systemically train and remind doctors of their obligations regarding conflicts of interest," he says. "Because of all that, we like to say our platform is a terrible place to buy insider information. But if someone chooses to do bad things, systems and controls can only mitigate that risk."

State sunshine laws that apply to his university require him to disclose all relationships with drug companies, Dr. Y says. "But I don't think anybody discloses their nonpharmaceutical contacts," he says, a category that would include payments from GLG.

Dr. X says that he's not trying to hide any financial activities. In fact, he doesn't even buy stock in individual companies—"the market is illogical, for starters," he explains. But he requested anonymity because he wasn't sure how his university would react if it knew he was working for GLG. And even as a GLG expert, he generally avoids conversations with stock analysts, preferring to talk with a company developing a particular technology or a venture capitalist looking for background information.

Staying on the right side of the law is easy for him, Dr. Y says, "because I'm risk averse and I'm afraid of getting into trouble." He says his work is preclinical, and he doesn't take money from companies developing drugs in his field, which he says makes him a

rarity among researchers he knows.

His GLG consulting isn't an issue for his institution, he adds: "They just want to be sure what I do doesn't affect my research." Even so, Dr. Y insisted on anonymity because of concern that something he said might offend GLG, which controls the flow of potential clients.

For Dr. Z, anonymity is all about avoiding publicity. "I'm still young, and I don't want anything to mess up my career," he says.

—JEFFREY MERVIS

LETTERS

edited by Jennifer Sills

Creating a Nobel Culture

IN THEIR PERSPECTIVE "A GOLDEN ERA OF NOBEL LAUREATES" (23 November 2012, p. 1033), J. L. Goldstein and M. S. Brown call attention to nine physicians who trained at the National Institutes of Health (NIH) in fundamental research from 1964 to 1972 and were subsequently awarded Nobel Prizes. They speculated on factors responsible for this remarkable confluence, contrasting current research and medical education to their experiences. As dean of a research-intensive medical school who also trained at NIH, I offer some alternative perspectives.

The NIH was a magical environment for biomedical research during the 1960s and 1970s. Many young physicians seeking academic careers—and some also wishing to avoid the military draft—spent time in NIH laboratories, some in combination with specialty training. The scientific environment was intoxicating, research resources seemed virtually unlimited, and scientific excellence was the highest value. Many experienced research success and now populate the leadership of biomedical research and academic medicine.

Goldstein and Brown also raised important concerns. The first relates to the tension between basic and translational research. The authors point out that whereas many of their later discoveries had clinical impact, they were not conducting "translational research" while at NIH. Few would contest that important biological research need not be translational. Fundamental inquiry into molecular, cellular, and physiologic pathways is foundational to translational and clinical research.

Nevertheless, many who launched research careers at NIH did research aimed at understanding disease. The NIH Clinical Center was a hotbed of bedside-to-bench research. Patients with rare and complex disorders stimulated basic investigations of molecular pathways with mentors in labs only a hallway away.

We spend too much time today unproductively parsing the definitions and relative value of basic and translational research. All research should be assessed by one criterion: the novelty, importance, and impact of the insights generated. Less important are the motivation of the scientist (e.g., understanding molecules and cells versus understanding disease), whether research links to health, the model system used (e.g., single molecules, mice, or humans), and the department in which it is conducted.

A second issue involves the status of research in medical education today. The authors state that while basic science was core to medical education in the 1960s, science is presented in an abbreviated manner today. I do not recognize this as an accurate description of science education in today's medical curricula, at least at research-intensive medical schools. Since the 1960s, the knowledge base within biomedical science has exploded, while important new areas such as health policy, global health, and biomedical ethics have emerged, requiring curricular choices. MD-Ph.D. programs train



physician-scientists, and many schools now require scholarly projects of all students.

The authors state that in the 1960s, the "best and the brightest" graduates were expected to pursue research careers. We delight when students pursue research, as many do today. We are also delighted when the "best and brightest" pursue clinical care and innovation, education, global health, and policy. Biomedical science is critical to the development of new treatments, but is one of several core missions of medical schools, as reflected in the diverse choices that medical students make.

Goldstein and Brown are concerned that NIH has shifted focus from basic science and "curiosity-driven" research to translational research and "big science." I have considerable sympathy for this view. Research into fundamental mechanisms is critical, and selectively diminished support for such research threatens the scientific enterprise and human health.

The development of consortia and "big data" approaches arose from the hypothesis that they enabled discoveries otherwise impossible. Some, such as the human genome project, have fulfilled much if not all of their promise. With others, the jury is still out. The balance between individual curiosity-driven research into basic mechanisms and disease pathogenesis versus big science consortia will evolve, though it's virtually certain that both will be necessary.

The nine Nobelists are products of an NIH program in which physician trainees were instilled with passion for fundamental research, and the opportunity for medical students and physicians to be exposed to and inspired by great science is as important now as it was then. But the many opportunities and challenges facing medicine and research

Letters to the Editor

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today suggest that a more complex solution will be needed. The path forward will determine our success in both biologic discovery and in applying these discoveries to improve the health of the population. **JEFFREY S. FLIER**

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Antarctic Treaty System Past Not Predictive

IN THEIR LETTER “ANTARCTIC TREATY SYSTEM ready for a challenge” (2 November 2012, p. 603), M. Haward *et al.* respond to the Policy Forum that I and my coauthors wrote, “Challenges to the future conservation of the Antarctic” (13 July 2012, p. 158). Haward *et al.* express optimism about the Antarctic Treaty System (ATS) and argue that the actions of Antarctic Treaty Parties and the effects of those actions were misconstrued in the Policy Forum. Their optimism and criticism are misplaced.

First, the ATS, via the Commission for the Conservation of Antarctic Marine Living Resources (CCAMLR), is struggling to manage the Antarctic toothfish fishery (1) and to establish a marine-protected area in the Ross Sea region, considered by many to be among the world's last great wildernesses (2). Despite CCAMLR's past successes, these recent developments signal a shifting global context for Antarctic conservation and resource management—one that is proving much more challenging than in the past, as the Policy Forum made clear.

Second, Haward *et al.* ignore further signs of interest in Antarctic resources and ongoing political maneuvering to secure its future potential use. For example, the Russian Federation has previously signaled its intention to strengthen economic capacity through investigations of Antarctic mineral, hydrocarbon, and other natural resources, and has done so again at the most recent meeting of the Antarctic Treaty Consultative Parties (3). The International Association of Antarctica Tour Operators has further projected resumption of the rise in tourist numbers to the region, despite a previous slowdown (4). In focusing on the historical successes of the ATS,

Haward *et al.* unwisely downplay accumulating evidence of rapidly changing demands on and aspirations for the region, and widespread concerns about growing difficulties with the management of this global commons (5–7). Clearly, the ATS is well placed to address Antarctic governance challenges. Position and past successes, however, provide no guarantee of future efficacy. **S. L. CHOWN**

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Sustaining China's Water Resources

CHINA'S WATER CRISIS IS RECEIVING WORLDWIDE attention (“Water sustainability for China and beyond,” J. Liu and W. Yang, Policy Forum, 10 August 2012, p. 649). The need for coordinating multiple government agencies has been highlighted, but involvement at both the corporate and the grassroots level is urgently required to ensure sustainability of the country's water resources.

Many of the world's largest multinational companies rely on Chinese water suppliers. Information technology (IT) companies use water of higher standard than Chinese drinking water, but the same companies' wastewater discharge delivers high concentrations of heavy metals into rivers and lakes (1). Several nongovernmental organizations implicated a Chinese supplier of an international IT company in heavy metal contamination of a lake that discharges into the Yangtze River, causing sediment copper concentrations to rise to more than 100 times the NOAA standard (2). Lack of involvement and commitment of large international companies in wastewater

treatment will exacerbate water pollution in China. Yet the private sector can and should play an important role in tackling global environmental challenges (3).

Grassroots involvement is a prerequisite for galvanizing and developing action for China's water sustainability. Awareness of water pollution problems among the growing middle class in Chinese urban areas has expanded rapidly in recent years (4). Protests about water pollution threats in the cities of Xiamen, Dalian, Shifang, and Qidong forced local governments to curtail industrial developments. *Weibo* (Chinese Twitter) effectively highlighted the potentially disastrous pollution from tourism development at Yamdrok Lake, a holy lake in Tibet (5). The online campaign to “Save Yamdrok Lake” received massive national support. Consequently, local government and the development companies were forced to abandon the plan, although such projects could still be relocated elsewhere (6).

Collaboration between Chinese government agencies, corporations, and grassroots organizations will promote water resource sustainability most effectively.

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CORRECTIONS AND CLARIFICATIONS

Perspectives: “Integrating the captive and the wild” by K. H. Redford *et al.* (30 November 2012, p. 1157). The bird labeled “peregrine falcon” in the figure on page 1158 is in fact a northern goshawk (*Accipiter gentilis*). The figure has been corrected to show a peregrine falcon in the HTML and PDF versions online.

Reports: “Identification of a universal group B *Streptococcus* vaccine by multiple genome screen,” by D. Maione *et al.* (1 July 2005, p. 148). Coauthor Vincenzo Nardi-Dei's name was missing the hyphen. The name has been corrected in the HTML version online.

ECOLOGY

It's a Wonderful Gift

Rolf O. Peterson

It is not often that mathematical theory is tested with a machine gun. In 1981 Dave Kelleyhouse, a wildlife biologist employed by the state of Alaska, submitted a purchase requisition for an automatic rifle to shoot wolves from aircraft in order to increase moose hunter success. If removing wolves was the management goal, it seemed to make sense to accomplish this as efficiently as possible. "Machine-gun Kelleyhouse"



didn't reckon that public opinion would play a role and that, once the public weighed in, his supervisors would be displeased (1).

It is doubtful that elegant math was really on the wildlife biologist's mind. But 11 years later, when the "experimental results" of killing wolves entered the scientific literature, it was packaged as a field test of a theory based on very seductive mathematical equations and graphs. The idea was that, if wolves and wolf kill rates could be brought to a very low level, low-density moose populations would increase and then remain at high density even when wolves were allowed to recover (2). Alas, the general result of temporary, intensive killing of wolves did not result in abundant moose, much less a "Serengeti of the North" (3). Instead, in almost all cases, as wolves recovered, there was a proportional reduction in moose density.

Proportional change in predator and

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prey populations is the theme of *How Species Interact*, the slimness of which belies its actual importance. In it, theoretical ecologists Roger Ardit (AgroPar-isTech) and Lev Ginzburg (Stony Brook University) provide a comprehensive summary of their long journey to recast scientific understanding of predator-prey dynamics. The authors continue to nudge ecologists beyond the classical Lotka-Volterra paradigm to a more realistic view they have termed "ratio dependence." Although their argument seemingly focuses on a small, arcane arena of population biology, few areas within ecology could be more fundamental (4).

Over two decades ago, Ardit and Ginzburg published a provocative paper in which they argued that ecologists had been misled during the previous half-century (5). Notwithstanding the elegant experimental work by Gause (6), Holling (7), and others in the decades following the much-cited work of Lotka (8) and Volterra (9), Ardit and Ginzburg contend that theories of coupled predator-prey dynamics rest on an incorrect premise: that predator kill rate is a function of prey density. Their contrarian view is that predator kill rate is best understood in relation to the ratio of prey to predator. At a fundamental level, predators represent a "conversion" of biomass from their prey, so logically one would expect a proportional relationship.

It would be a vast understatement to say that prey-dependent models based on Lotka-Volterra dynamics are the entrenched, majority view, for they are the foundation of most of the scientific literature on predation over the past century. Never mind that the outcome of the basic Lotka-Volterra model is very sensitive to initial conditions; that the only model outcome, if the parameters are carefully adjusted, is an everlasting predator-prey cycle; and that no serious student of predation believes the model suitably depicts the real world. For over two decades, Ardit and Ginzburg have picked away at the reigning

How Species Interact Altering the Standard View on Trophic Ecology

by Roger Ardit and
Lev R. Ginzburg

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paradigm, all the while championing their alternative ratio-dependent model. Amassing compelling evidence from mathematics, logic, field data, and experimental studies, they have gradually gained support.

Only dedicated specialists will have closely followed the debate over ratio dependence.

The present book usefully distills the theory in a grand narrative, albeit one written by the challengers to prevailing opinion. Ardit and Ginzburg have studied carefully the arguments of their critics and respond with a hypothesis based on "gradual interference" along a gradient of predator density. By this notion, predators that exist at moderate to high density interfere or indirectly compete with one another, leading to a "reduction in consumption rate due to sharing available prey with their neighbors." Perhaps satisfyingly, gradual interference accommodates both prey-dependent and ratio-dependent perspectives, each operating in pure form at the extremes of predator density.

After a career in field biology that's led to an appreciation for the inherent complexity of predator-prey dynamics, I admit to being impressed by the immediate usefulness of viewing predation through ratio-dependent glasses. Elegant mathematics that describes the essential core dynamic is a "wonderful gift" to our understanding of the natural world, which physicist Eugene Wigner said "we neither understand nor deserve" (10). Imagine reaching the following insights through mathematics and critical thinking: Whereas the presence or absence of predators greatly affects prey numbers, annual variation in predator density does not. Killing a wolf will not likely improve moose-, elk-, or deer-hunting success for humans. Improving habitat will increase prey numbers more successfully than will controlling predators. Biological control of insect pests can actually work, even in a (mainly) ratio-dependent world. The mutual dependency of predator and prey is fundamentally asymmetrical—prey matter to predator populations far more than vice versa.

In reviewing the progress in scientific understanding of predator-prey systems, Ardit and Ginzburg decry the gap that exists between theory and application. Microbiologists quickly confirmed and accepted the basics of consumer-resource dynamics, but many applied ecologists (e.g., in my own field of wildlife management) have an unfortunate phobia for all things mathematical. Readers

who do not fully understand the equations of Arditi and Ginzburg will not appreciate all of the elegance and evidence in *How Species Interact*. Yet all ecologists will certainly gain by grasping the conclusions and philosophy found in the book.

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HUMAN DEVELOPMENT

Minding the Gap

Johan J. Bolhuis

Cambridge ethologist Patrick Bateson once started a lecture on the nature-nurture debate suggesting that “There are two kinds of people: those who believe in dichotomies and those who don’t.” His comment highlights an important aspect of the debate, which is actually not about nature versus nurture but whether behavior can be dissected into “innate” and “acquired” components. Famously, behavioral biologist and Nobel laureate Konrad Lorenz suggested that that was the case. In the 1950s and 1960s, he and psychologist Daniel Lehrman engaged in heated debates that are now among the classics of the behavioral biology canon. Lehrman criticized Lorenz for his dichotomous thinking and suggested instead that development is a complex process involving continual interaction between the individual and its environment. Because the environment changes all the time, the nature of the interaction itself also changes during development (1). Subsequently, the term “innate” became anathema to most biologists, whereas until not so long ago (2) it was still used quite happily by many psychologists.

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I would have thought that Lehrman had finally settled the matter, but apparently developmental psychologist Dale Goldhaber (University of Vermont) begs to differ. I was quite surprised to see that in *The Nature–Nurture Debates*, he does not refer to the classic Lorenz–Lehrman discussion at all. When reading his interesting historical account, however, I realized that the real dichotomy here is between biology and psychology. Whereas for biologists like myself, Lehrman has settled the matter, allowing us to move on, Goldhaber makes quite clear that the debates continue among psychologists.

Futhermore, what Goldhaber calls the classic debate was actually going on long before Lorenz and Lehrman had a go. Of the many participants, I was particularly impressed with the insights of Anne Anastasi, who seems to have independently reached similar conclusions to Lehrman at around the same time. Goldhaber’s historical account offers some interesting surprises. One would have thought that Edward Thorndike, who formulated a number of laws of learning, would be an ardent empiricist. But Goldhaber reveals that Thorndike actually had strongly held nativist beliefs that are difficult to distinguish from blatant eugenics, expressing considerable pessimism as to the power of education in improving mankind.

In addition to the classic debate, Goldhaber identifies “new” and “proxy” debates that are continuing. Beyond the rearguard battles, however, scientific practice has moved on. For instance, in developmental cognitive neuroscience, current theories of the development of face recognition involve complex interactions between learning and predispositions subserved by subcortical mechanisms (3). Likewise, it has become apparent that the remarkable ability of children to acquire language is the result of a complex interplay between domain-general learning abilities and universal grammar (4). Perhaps what ultimately made the old dichotomous thinking obsolete is the recent epigenetics revolution, exemplified by the work of Michael Meaney and colleagues (5), which Goldhaber discusses extensively in the framework of developmental systems theory.

In the final chapters, Goldhaber attempts to “bridge the gap” by invoking evolutionary psychology. I wish he hadn’t. This is trying to solve one controversy by introducing another. Essentially, the author suggests we need a synthesis of evolutionary psychology

and developmental systems theory to resolve the debates. I feel that developmental systems theory is more than adequate to analyze development, and evolutionary psychology is completely out of place here. Goldhaber magnanimously acknowledges that his solution is not original but has been around in essence since the 1970s. Thus it would seem that the problem—if indeed there is one—has been solved long ago. The author suggests that “it doesn’t hurt to be reminded of things once in a while.” He may have a point there, and I certainly enjoyed reading his historical account of the various debates.

But I fear that Goldhaber’s message will not reach the audiences that need it most: evolutionary psychologists and other scientists who cling to a reductionist nativist view of development and, most important, the general public. Unfortunately, the truth about development as a dynamic process cannot be captured in simple sound bites. It is so much easier to boldly state that a particular human trait lies “in our genes”—which is why the media continue to use such outmoded terminology. I share the frustration of many colleagues when our students continue to talk about “innate behavior” despite our attempts

to provide them with a nuanced view of development. This sorry state of affairs often forces developmental scientists to fight ever-popular notions of nature and nurture. For example, geneticist Simon Fisher (a codiscoverer of the FOXP2 gene that is mutated in the members of an English family with severe speech problems) spends considerable time in his public talks and reviews (6) stating emphatically that FOXP2 is not a “language gene.” In fact, there is no such thing as a gene for any kind of behavior or cognitive mechanism. So maybe there still is work to be done—and gaps to be bridged. Goldhaber has indicated ways of doing so but regrettably favors a less useful one himself. Nevertheless, his book offers an excellent historical introduction to the debates and as such deserves a wide readership among biologists and psychologists alike.

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The Nature–Nurture Debates

Bridging the Gap

by Dale Goldhaber

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Regulation of Online Social Network Studies

R. Benjamin Shapiro¹ and Pilar N. Ossorio^{2,3,4*}

Research conducted on social networking sites (SNSs) offers scientists the opportunity to understand human social behavior and to use SNSs for experimental interventions, such as increasing civic participation (1). The number of studies and interventions conducted in SNS environments is growing. Unfortunately, U.S.-based academic researchers and institutional review boards (IRBs) have received no guidance from the Office for Human Research Protections (OHRP) regarding how to apply human subjects regulations to SNS research (2). Here, we address two ethical and regulatory challenges: (i) whether adolescents who participate in such research through commercial portals, such as Facebook, should be categorized as children for regulatory purposes; and (ii) the extent to which researchers may collect data about SNS participants' Facebook "friends" (FBFs).

Online educational games have been used and studied in controlled classroom settings (3), but researchers are now creating games open to all SNS users (4). By positioning players as protagonists who identify and solve problems while interacting with each other, game components, and environments, virtual games can stimulate scientific reasoning as well as increase motivation to learn (5, 6). Several academic institutions are conducting design-based research on SNSs with federal, state, and nonprofit funding. In our pilot study, middle-school girls who played a science education game became more likely to believe they could become physicians (7).

Consent and the Adolescent Player

University researchers studying players of SNS games are engaged in human subjects research and require either IRB approval or exemption, as well as players' informed consent or assent (2). Is parental permission required for researchers to study adolescent players? [Adults give consent for their



own research participation; parents give permission, rather than consent, for their children's research participation (8).] Some commentators on SNS research have considered adolescents to be children and have stated that parental permission will usually be necessary for such research (9, 10), but we disagree.

The Common Rule does not specify a particular age below which a person is treated as a child. Rather, it defines children as: "Persons who have not attained the legal age for consent to treatments or procedures involved in the research..." (8). This definition coordinates the Common Rule with other applicable laws, such as state statutes permitting adolescents to consent to treatment for sexually transmitted diseases. Applicable to the SNS research, the federal Children's Online Privacy Protection Act (COPPA) defines children as persons under the age of 13 (11). COPPA prohibits commercial operators of online services (such as Facebook) from engaging in unfair or

How should research studying adolescent players of online educational games be conducted responsibly?

deceptive practices with respect to the collection, use, or disclosure of personal information from and about children (12–14). Facebook and other SNSs have responded to COPPA by prohibiting access to their services by people under 13 (15). Although some parents help their children evade age limits, SNSs delete accounts identified as belonging to underage users (15).

We believe COPPA and the Common Rule together indicate that IRBs should allow adolescents who enter an experimental game through a Web site that limits access to people 13 or older to consent on their own to research involving collection of information about their gaming activities. This approach is not a waiver of the requirement for parental permission. Rather, we argue that people 13 and older should not be categorized as children for IRB review of SNS research. In the nonresearch context, adolescents can legally use SNSs to play games and to provide identifiable, private information about themselves, so they should be able to consent in the research context.

Parents who monitor their adolescents' Internet activities can observe the consent process and read the information, and parents are always free to prevent their adolescents' participation in IRB-approved research. IRBs may also impose additional protections for vulnerable populations (16), such as provision of age-appropriate postplay debriefing materials or even game features that could detect player distress and respond to it by modifying the game environment.

Furthermore, researchers should use the online environment to deliver innovative, informative consent processes to participants in online research. This is especially important given the general public's ignorance about data collection over the Internet. For instance, although 44% of American parents "are extremely or very concerned that their children might have information about them used for targeted advertising," only 9% of parents whose children use SNSs believe their children's data have been used in this manner (17). In reality, 100% of SNS participants have their data mined for targeted advertising.

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Collecting Data on Friends

SNS research also raises regulatory and ethical questions about collecting information on members of participants' social networks. One of our research questions is whether SNS science education games can help overcome race and gender disparities in science education. To learn how biases shape game participation, one might examine the demographic characteristics of FBFs whom research participants invite to play compared with the demographics of their FBFs generally. The average Facebook user has 130 FBFs (4); it is not plausible that all would consent to data collection. Requiring consent from nonplayers would introduce statistical bias and diminish research rigor.

Creators of Facebook application programming interfaces (APIs) have access to identifiable information about game players' FBFs, much of which is necessary for providing game-user interfaces. Should researchers who use APIs be required not to store identifiable information on nonplaying FBFs? Should researchers be required to write applications in ways that do not allow them access to FBFs' information, but that utilize participants' browsers to produce deidentified aggregated information?

Perhaps the closest analogy involves collection of family history information from research participants. Although researchers have engaged in this practice for decades, it became controversial in 2001. Regulators temporarily halted all human subjects research at Virginia Commonwealth University after a research participant's father objected to collection of sensitive family history information (17). Commentators and regulators emphasize that researchers who collect identifiable private information about relatives are engaged in human subjects research on the relatives and must obtain their consent, unless an IRB waives this requirement (9, 17). However, if the information is not identifiable, or is identifiable but not private, relatives' consent is not required (18).

Under the Common Rule, information is private if a person reasonably expects it will not be observed or recorded or if it is provided for a specific purpose and the provider reasonably expects it will not be made public (2). Social conventions regarding information privacy on SNSs are still developing, so people's expectations of privacy may be in flux.

Facebook warns users that some information is "always publicly available," including name, profile picture, network, user name, user identification, and any information one

chooses to make public (19). State and federal courts have held that individuals do not have a reasonable expectation of privacy in information posted to a SNS (20); however, the issue is new enough that courts have not yet considered many aspects of online privacy. In this socio-legal context, IRBs are justified in treating some identifiable information about FBFs as "not private," so its collection and use would not constitute regulated human subjects research. Even in the absence of regulation, however, investigators have ethical duties to minimize risks to people whose data they use. For instance, investigators must maintain adequate data security. They must ensure that published results do not allow for easy reidentification of participants or FBFs.

Investigators and IRBs must also determine that research risks are reasonable in relation to anticipated benefits (2). We believe many SNS interventions are minimal risk. However, some will not be. One could imagine, for instance, games attempting to influence mental health that could present substantial psychosocial risks. SNS interventions could generate embarrassing data about identifiable participants' behaviors and biases. Balanced against such risks are potential benefits to society, including improvements in students' learning, self-efficacy, and motivation; knowledge about motivating individually and socially beneficial behaviors, such as exercise or civic participation; and the creation of new forums for engaging informed publics in scientific discovery and policy.

Design-based SNS studies raise other ethical issues not covered here. Researchers, ethicists, regulators, and funders need venues for discussing these issues and collaboratively developing ethics guidelines. IRBs have been criticized for inconsistent assessment of social, psychological, and economic harms and benefits in other research contexts (21) and will need guidance for assessing SNS research. IRBs must also be educated about technical options for addressing ethical issues, and game designers must be educated about ethical and regulatory issues to facilitate further development of such technical options.

OHRP recently circulated a proposal to revise the Common Rule (21). The proposal mentions the Internet as an emerging experimental sphere; however, it contains no discussion of how research in this sphere ought to influence regulatory revisions or how such research would be reviewed under the new proposals. In the revisions, OHRP takes the position that all informational risk in

research can be minimized by adequate data security. Although we believe data security is important, we suggest that research design also affects the risk calculus.

Lack of ethical guidance can stymie academic SNS research, potentially rendering academia irrelevant to an important domain of human activity. The private sector is charging ahead, creating de facto standards for data use that default to allowing commercial enterprises broad access to personally identifiable information and behavior. The goals of most academic research surely have as much social value as selling more products to SNS users. Providing marketers greater access than academic researchers to people's online information would be an ethically dubious outcome.

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How to Solve Protein Structures with an X-ray Laser

John R. Helliwell

Femtosecond pulses from an x-ray laser are used to solve the room-temperature structure of a protein.

For over a decade, biologists have asked whether x-ray lasers can be used to determine the structures of biomolecules such as proteins. Such methods have the potential to allow structure determination from micro- or even nanoscale crystals, but radiation damage can be extensive and data interpretation is fraught with difficulty. On page 227 of this issue, Redecke *et al.* (1) overcome these problems to determine the room-temperature structure of a protein of importance to drug discovery.

The authors studied *Trypanosoma brucei* cathepsin B (TbCatB), a protein that is important in structure-based drug discovery aimed at eradicating sleeping sickness. They first grew protein microcrystals with a typical volume of just $\sim 4 \mu\text{m}^3$ [see the figure (A)] in insect cells. The crystals were delivered in a liquid stream that flowed past repeated 42-femtosecond x-ray pulses [see the figure (B)]; the flow was asynchronous, reducing the efficiency of an x-ray flash hitting a crystal. Nearly 300,000 microcrystals with a combined crystal volume of 100 by 100 by 100 μm^3 were used (a volume that is quite typical for single protein crystals used in structure determination). The total x-ray exposure time, with allowance for misses, was only 126 nanoseconds. The authors used a known structure of a similar protein to solve the new structure (a method called molecular replacement).

Synchrotrons have long been used to interrogate micrometer-scale protein crystals. Early tests showed that protein crystals with diameters of 20 μm would yield sufficiently strong diffraction data to allow structure determination (2), which led to the establishment of microbeam instruments at many synchrotrons (3). Use of such synchrotron

x-ray beams with diameters of about 10 μm has also become very popular in chemical crystallography (4). The current European Synchrotron Radiation Facility upgrade program (www.esrf.fr) is making x-ray beams with even smaller, submicrometer, diameters more readily accessible.

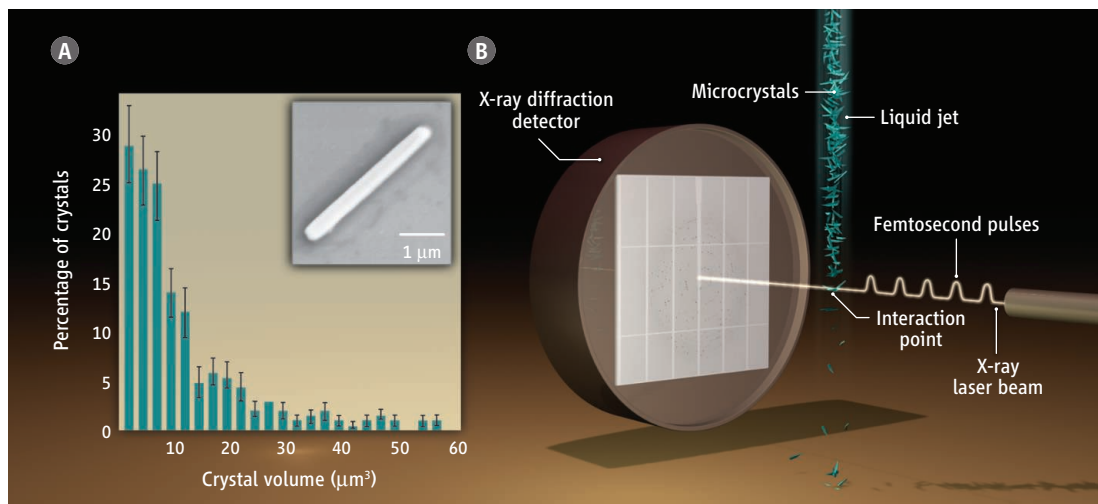
X-ray laser sources deliver the same intensity within tens of femtoseconds that a typical synchrotron beamline delivers in a millisecond. The short, intense pulse of the x-ray laser allows radiation damage mechanisms that occur on time scales longer than tens of femtoseconds to be avoided. Furthermore, the exceptional brightness of the laser reduces the required sample volume by several orders of magnitude. However, there have been many questions regarding data interpretation. For example, would useful intensity be measurable from the diffraction pattern over all the usual scattering angles? Would there, at much higher scattering angles, be details of femtosecond time-dependent changes in the x-ray scattering factor? What about the ejection of core shell electrons (5), which could potentially ruin anomalous scattering phasing? Would the sample withstand the x-ray beam both thermally and in terms of overall and specific radiation damage?

Redecke *et al.* now show that useful electron density details can be derived from an

x-ray beam diffracted by tiny single crystals. Time-dependent changes to the x-ray scattering factor may still be hidden and may emerge when a sample is used that diffracts to higher scattering angles.

The authors used a focused x-ray beam with a diameter of 4 μm , reasonably well matched to the crystal lengths of up to 10 μm but quite a lot bigger than the cross section (~ 1 by 1 μm^2). A smaller focus would be needed for crystals in the submicrometer size range. Also, the x-ray wavelength was 1.32 Å, but could, in principle, be shifted to a longer wavelength, which would give a gain in sample scattering efficiency (6). The combination of these useful gain factors means that protein structure determination from protein nanocrystals, which have a smaller x-ray scattering strength than do microcrystals, is within reach of protein x-ray crystallography.

To get a full view of the protein structure, single crystals are conventionally rotated in the x-ray beam. Redecke *et al.* instead measure many tiny crystals, each of which is in a different orientation. This serial method leads to a myriad of still diffraction patterns. Each x-ray flash destroys the tiny crystal that is hit owing to the intense x-ray power. In addition, each diffraction pattern can be of limited statistical quality—that is, it has a low number of counts per diffraction spot. Therefore, a



Tiny crystals, big result. (A) The majority of crystals used by Redecke *et al.* had a volume of less than 10 μm^3 . (Inset) A typical in vivo-grown crystal used in the study. (B) Irradiation of a stream of microcrystals with femtosecond x-ray pulses enabled determination of the structure of a protein that is important for drug discovery. [Adapted from (1)]

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large number of crystals are needed, although it may be possible to use fewer than the current study did.

To determine the crystal structure, the authors used the conventional crystal structure of solubilized and recrystallized TbCatB (Protein DataBank code 3MOR), determined at 100 K (7). The new structure provided additional structural details at room temperature and provided higher resolution (2.1 Å, compared with 2.5 Å for 3MOR). This extra detail is potentially important at the physiological temperatures at which a drug must act (8). Detailed comparison of the TbCatB crystal structure (1) with the equivalent enzyme from humans (9) revealed structural differences that may be important for drug design, because any drug targeting the TbCatB protein should not affect the human enzyme.

Another distinctive feature of the study is the use of femtosecond x-ray pulses. To date, studies of time-resolved evolution of small changes in a protein crystal structure extend into the picoseconds regime (10). The use of x-ray lasers should now allow such studies to be taken into the femtosecond regime.

There will be very wide interest in this result from the structural biology and structural chemistry research communities as well as pharmaceutical companies, which are frequent users of synchrotron sources. As demonstrated with this successful application reported by Redecke *et al.*, x-ray lasers are set to enable protein structure determination of proteins that are difficult to crystallize. Getting next into the nanometer crystal-size range looks doable and exciting. The fact that the structure reported by the authors may aid

the discovery of drugs against sleeping sickness is an important added bonus.

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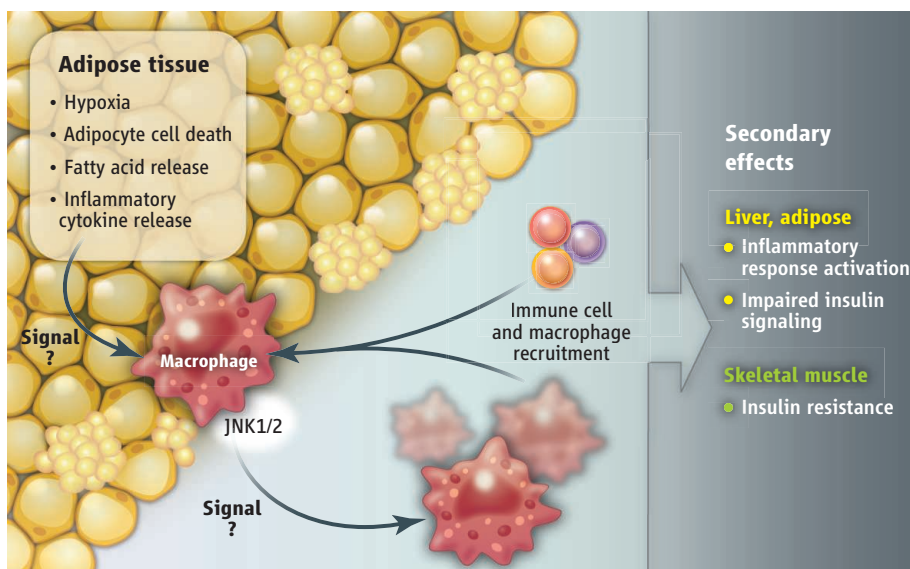
BIOMEDICINE

Improving Metabolism by Throwing Out All the JNK

Anthony W. Ferrante Jr.

Obesity activates a complex immune response that includes the production of proinflammatory molecules and the recruitment of immune cells to key metabolic organs including adipose tissue (1, 2), liver (3), pancreas (4), and hypothalamus (5). This response has been implicated in derangements of local tissue metabolism and the subsequent development of obesity-associated disorders—especially type 2 diabetes, nonalcoholic fatty liver disease, and dyslipidemia. Defining regulators of obesity-induced activation of the proinflammatory response remains a challenge that offers the potential to identify therapeutic strategies to treat several metabolic diseases. On page 218 of this issue, Han *et al.* (6) have established that signaling by the enzymes c-Jun NH₂-terminal kinases (JNK1 and JNK2) in myeloid cells is required for obesity-induced immune cell recruitment, inflammation in adipose tissue, and the development of insulin resistance and impaired glucose homeostasis.

Almost a decade ago, Hirosumi *et al.* found that obesity increases JNK activity in adipose tissue and liver of mice (7) and hypothesized that JNK1 and JNK2 are part of



Obesity and inflammation. In obesity, adipocyte hypertrophy and hyperplasia create the conditions shown, which trigger the activity of JNK1 and JNK2 in resident macrophages. This activation is required for subsequent immune cell recruitment to the adipose tissue, leading to a complex inflammatory response. Many of the secondary systemic metabolic sequelae also depend on JNK activation in adipose myeloid cells.

a cell signaling cascade that senses metabolic stress and activates obesity-induced inflammatory responses. Although deletion of JNK2 in mice had no discernible metabolic effect, mice genetically deficient in JNK1 were protected from weight gain in both dietary and genetic mouse models of obesity

(7). However, it was neither clear whether deficiency of JNK1 had beneficial metabolic effects independent of its effects on weight, nor in which cells' populations JNK activity contributed to adverse metabolic signaling.

Studies to determine whether JNK1 deficiency reduced obesity-induced inflamma-

tion and insulin resistance without affecting fat mass were inconclusive. The absence of JNK1 in hematopoietic cells did not alter weight gain in a dietary mouse model of obesity. However, some studies showed JNK1-dependent effects on obesity-induced inflammation and insulin resistance (8), whereas others did not (9, 10). But these studies were complicated by two factors: (i) the use of bone marrow chimeras, in which irradiation reduces the development of obesity, and (ii) the potentially redundant role of JNK2.

Han *et al.* genetically engineered mice that lack both JNK1 and JNK2 in myeloid cells and confirmed that the absence did not alter the adiposity of mice fed high-fat diets (although there was a small reduction of lean mass). By contrast, the absence of JNK1 and JNK2 in myeloid cells prevented obesity-induced increase in adipose tissue macrophages and inflammation. There were similar effects in reducing obesity-induced hepatic inflammation (albeit hepatic myeloid cells were not analyzed). There was no measurable effect on other myeloid cell populations, including eosinophils and neutrophils.

Han *et al.* further found that in mice made obese by a high-fat diet, the absence of JNK1 and JNK2 in myeloid cells normalized the response to glucose and insulin challenges and improved all measures of insulin sensitivity. Even in lean mice in which JNK deficiency did not alter fasting, circulating glucose, or insulin concentrations, there were slight increases in insulin sensitivity. JNK deficiency in myeloid cells did not improve all metabolic phenotypes in obese mice; there was no effect on circulating fatty acid concentrations or reduction in hepatic triglyceride content, although there was a trend for less hepatic steatosis.

In contrast to Han *et al.*, other studies revealed that deletion of only JNK1 in adipose tissue improves hepatic insulin sensitivity in obese mice but does not improve overall glucose tolerance or whole-body glucose disposal. Taken together, the earlier studies and that of Han *et al.* suggest that the obesity-induced stress response in adipose tissue depends in part on JNK1 in adipocytes, but the full pathologic metabolic effects depend on myeloid cells. The deletion of both *Jnk1* and *Jnk2* in mouse adipocytes should clarify whether adipocyte JNK has liver-specific effects on glucose homeostasis and the development of fatty liver disease.

How does JNK signaling fit into our emerging understanding of obesity-induced inflammation and insulin resistance? JNK appears to play several distinct roles. In non-hematopoietic cells, JNK1 is required for adi-

pose tissue expansion and the development of obesity (7). However, the normal adipose tissue mass in JNK1-deficient mice argues that JNK1 regulates adipocyte hypertrophy and hyperplasia indirectly, possibly by modulating the response of hypothalamic neurons to hormonal cues (9, 11). JNK signaling is also important in the development of insulin resistance in parenchymal cells; deficiency in JNK1 in hepatocytes reduces hepatic steatosis and insulin resistance (12).

In hematopoietic cells, JNKs serve partially redundant roles in controlling the immune response to obesity. The proximal signals in adipose tissue appear to be several: adipocyte death, excess release of fatty acids, hypoxia, stress to the endoplasmic reticulum, and a fibrosis-like response. Normalization of the macrophage content and of the inflammatory phenotype of adipose tissue by JNK deficiency in myeloid cells suggests that JNKs integrate these signals within macrophages to initiate inflammation and immune cell recruitment.

Although Han *et al.* more clearly define a role for JNK in mediating obesity-induced inflammation and insulin resistance, several important questions remain. The murine model of dietary obesity induces a particularly potent inflammatory response. Deter-

mining whether myeloid JNK signaling contributes to the immune response and insulin resistance to a similar degree in other obese animals and mouse strains will help establish whether there is a common signaling pathway required for insulin resistance. A working model of obesity-induced inflammation suggests that inflammatory signals impair insulin signaling in adipocytes; this increases basal lipolysis and leads to ectopic lipid deposition in muscle, liver, and pancreas. A more detailed analysis of lipid homeostasis should help clarify whether altered lipid deposition can explain, at least in part, improved insulin sensitivity in obese mice lacking myeloid JNK. These same mice will also be helpful in defining which immune cell populations deserve further study.

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10.1126/science.1233223

PHYSIOLOGY

When Metabolism and Epigenetics Converge

Paolo Sassone-Corsi

Nutrition, energy metabolism, and the plasticity of gene expression are linked through the action of epigenetic modifiers that are modulated by cellular metabolites.

Various cell types respond differently to the environment by using distinct circuits of genomic reprogramming. How does a fixed DNA blueprint allow flexibility in managing changes to environmental signals? Environmental inputs such as nutrition can modulate cell metabolism, and critical links between metabolism and epigenetic control—now widely thought to include chromatin remodeling, histone modifications, DNA methylation, and microRNA pathways (1)—are beginning to emerge (2, 3). Two

reports in this issue, by Shimazu *et al.* (4) on page 211 and Shyh-Chang *et al.* (5) on page 222, provide insights into this connection.

Histone posttranslational modifications, such as acetylation and methylation, occur at specific residues and, depending on their combination, have been associated with transcriptional activation and silencing, DNA repair, and recombination. The factors that elicit these modifications are enzymes that use metabolites as sources of, for example, acetyl or methyl groups, whose availability and intracellular localization may dictate the efficacy and specificity of the enzymatic reaction (2, 3). For acetylation, cellular metabolites such as acetyl coenzyme A (acetyl-CoA)

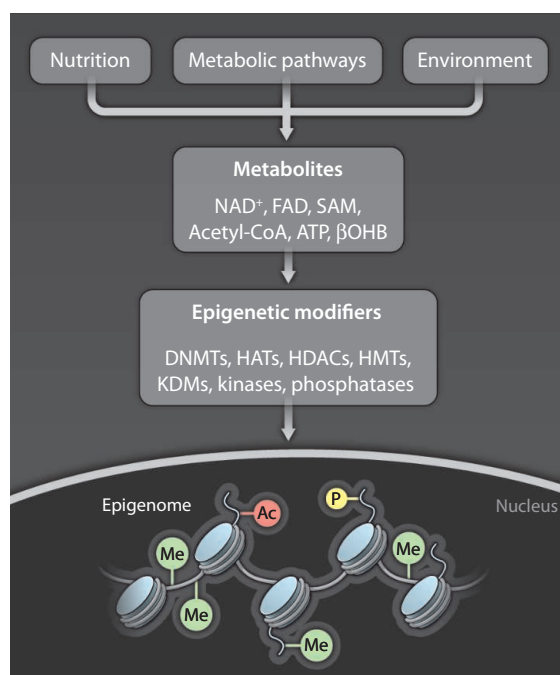
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and nicotinamide adenine dinucleotide (NAD⁺) regulate gene expression by serving as cofactors for epigenetic modifiers (2). For example, acetylation by histone acetyltransferases (HATs) depends on local subcellular acetyl-CoA concentrations (6, 7).

By contrast, deacetylation is mediated by histone deacetylases (HDACs). The class III HDACs are similar in structure to a yeast enzyme called silent information regulator 2 (Sir2). In mammals, the sirtuin family of HDACs (orthologs of yeast Sir2) is composed of seven members (SIRT1 to SIRT7), each with distinct subcellular localization. The key feature that differentiates these HDACs is their dependence on local NAD⁺ concentration for enzymatic activity, thereby linking their function to intermediary metabolism (8). SIRT1s have been thought to “sense” the beneficial effects of caloric restriction on physiology and have been associated with the control of mitochondrial energy metabolism, inflammation, aging, and tumorigenesis (8). However, their precise role

and underlying molecular mechanism(s) in controlling life span remains unclear. During fasting, cellular concentrations of NAD⁺ are high, and SIRT1 activity is elevated. Yet, when energy is in excess, NAD⁺ is depleted because the rampant flux through the glycolytic cycle promotes the conversion of NAD⁺ to its reduced form NADH (8). This notion directly links nutrition, energy metabolism, and epigenetic control. Among the HDACs, are sirtuins the whole story?

SIRT1s have been considered unique HDACs because of their dependence on an endogenous metabolite, whereas the function of all other deacetylases has never been directly linked to cellular metabolism. But indications that this may be possible did exist. For many years, researchers have used sodium-butyrate, a short-chain fatty acid that functions as a potent detoxifier of ammonia and neurotoxins, as a HDAC inhibitor. Butyrate induces cell cycle arrest, apoptosis, and differentiation in various cancer cells and causes accumulation of acetylated histones. Butyrate putatively functions by blocking substrate access to active sites in HDACs. Shimazu *et al.* noticed that the ketone body β -hydroxybutyrate (β OHB) is structurally similar to butyrate. β OHB may operate as



A plastic epigenome. DNA and histones are targets of multiple modifications that convey flexibility to the genome. These are elicited by modifiers whose activities are modulated by metabolites. The availability and subcellular compartmentalization of metabolites could contribute to the specificity of epigenetic control. Ac, acetylation; Me, methylation; P, phosphorylation; DNMTs, DNA methyltransferases; HMTs, histone methyltransferases; KDMs, lysine demethylases; FAD, flavin adenine dinucleotide.

a natural, endogenous HDAC inhibitor.

Ketone bodies are produced when fatty acids are broken down for energy, and their presence increases in individuals subjected to starvation and extended caloric restriction. Shimazu *et al.* found that β OHB acts as an endogenous HDAC inhibitor in cultured cells, leading to increased histone H3 acetylation at Lys⁹ and Lys¹⁴. It also activated the transcription of several genes controlled by the transcription factor FOXO3a (which has been associated with longevity in various organisms). These results are consistent with the model that elevated β OHB concentrations observed in mammals during fasting and caloric restriction contribute to resistance to oxidative stress observed under these conditions (9). Studies in the fly, worm, and yeast had implicated class I HDACs in the life span extension associated with caloric restriction (10–12), which suggests that conditions that increase β OHB concentrations, such as caloric restriction, might extend life span through the inhibition of class I HDACs.

Low-carbohydrate diets that induce ketogenesis, the generation of ketone bodies, are broadly neuroprotective and enhance resistance of neurons to oxidative damage induced by reactive oxygen species (ROS)

production (13). The results by Shimazu *et al.* indicate that the beneficial effect of such diets might be mediated by β OHB, causing increased gene expression of oxidative stress resistance genes. Calorie restriction and low carbohydrate diets reduce ROS production and improve mitochondrial function. That both calorie restriction and low-carbohydrate diets increase β OHB production could explain the overlapping biological responses that occur in response to both diets (14). Thus, the findings of Shimazu *et al.* seem to stress that SIRT1s may not be the only HDACs implicated in the longevity pathway and that metabolite-controlled histone acetylation is a widely used process.

Histone acetylation at Lys⁹ and Lys¹⁴ of H3 is often coupled to methylation at the Lys⁴ residue of the same histone tail, generating a permissive state for transcriptional activation (1, 2). If specific metabolites so profoundly influence histone acetylation, would histone methylation undergo a similar control mechanism? The main source of methyl groups in cells is S-adenosylmethionine (SAM). Shyh-Chang *et al.* have linked this question to the differentiation program of mouse embryonic stem cells (mESCs). The pluripotency capacity of mESCs requires the amino acid threonine (15), although how this metabolic requirement is translated into an epigenetic code required to establish pluripotency has been a mystery. Shyh-Chang *et al.* reveal that the balance between SAM and S-adenosylhomocysteine (SAH) correlates with H3 Lys⁴ trimethylation, whereas mono- and dimethylation at the same residue are less sensitive. Only a few methyltransferases are known to be involved in trimethylation at the Lys⁴ residue. Thus, it may be that mESC pluripotency depends on a restricted group of epigenetic modifiers.

An intriguing feature of this finding is that H3 Lys⁴ trimethylation appears to be more sensitive to changes in threonine metabolism than methylation at other lysine residues on H3 or other histones. One possible explanation is that Lys⁴ methylation is highly abundant and has a greater turnover when compared to other lysine residues. Alternatively, distinct metabolites may localize to chromatin subdomains, favoring the clustering of relevant posttranslational modifications at specific genomic loci. The presence of metabolite “niches” within specific chromatin subdomains has been proposed (2) and is conceptually intriguing when placed in parallel with the idea of nuclear subcompartments and transcription “hubs” (16). As connections between epigenetics and metabolism emerge, it may be possible to consider new pharmacological interventions for a variety of pathological conditions.

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MATERIALS SCIENCE

Water-Responsive Polymer Composites on the Move

Hyoki Kim and Sunghoon Kwon

As the power consumption of electronic devices continues to decrease, the amount of energy harvested from the ambient environment can be enough to drive functional circuitry. Harvesting such energy may be particularly advantageous in environment sensing and surveillance monitoring. For example, replacing the batteries for a wireless network monitoring hundreds of remote sensors would likely be difficult, and self-sustained systems could reduce maintenance costs. On page 186 of this issue, Ma *et al.* (1) report that the energy can be harvested from water gradients in the

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environment (e.g., a wet surface) by means of a specially designed composite film. The potential energy of a moisture gradient can be converted inside the composite actuator and stored as elastic potential energy, and then used to produce mechanical work or to create electricity.

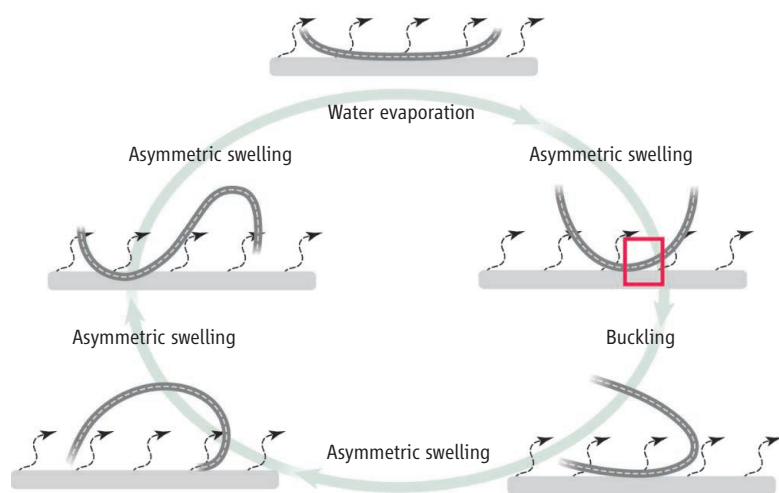
The conventional sources of ambient energy harvesting have been light, heat, thermal gradients, wind, acoustic noise, and electromagnetic waves (2–5). Previous attempts to make an actuator powered by a water gradient were based solely on the water responsiveness of anion-doped polypyrrole (6). Ma *et al.* made molecular networks of polypyrrole and a polyol (pentaerythritol ethoxylate, PEE) containing borate (see the figure). In this composite material, polypyrrole serves

Films swollen by wet surfaces curl up and store mechanical energy that can be converted into electricity for powering small devices.

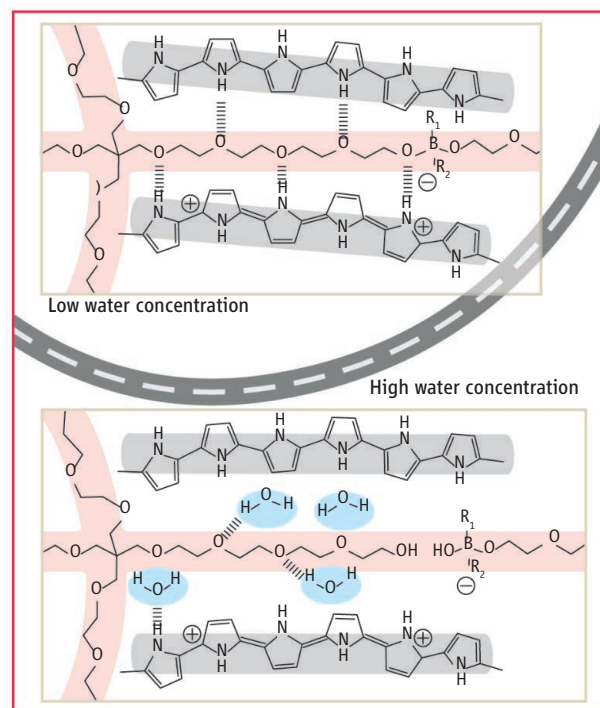
as a rigid polymer matrix that holds the elastic, interpenetrating polyol-borate network. The water responsiveness comes from the reversible hydrolysis and esterification reaction of polyol borate as well as from the strong hydrogen-bonding interactions between the polyol borate and rigid polypyrrole.

When this composite film was placed on a wet substrate, the bottom surface of the composite film swelled and the film curled. The contact area of the substrate and the film decreased and the center of gravity of the film rose, which caused the film to become mechanically unstable and “topple.” This sequential asymmetric swelling and toppling process led to continuous actuation of the film.

For various sensing applications, it is essential to convert mechanical energy to



Working when wet. Continuous locomotion of an actuator is illustrated. A composite material of polypyrrole (gray in inset) and a polyol (PEE, pink in inset, containing borate groups with side chains R_1 and R_2) moves continuously when powered by the energy from a water gradient (blue in inset). When the actuator is placed on a wet substrate, the bottom surface of the actuator absorbs more water than the top surface of the actuator. Continuous movement of the actuator is caused by the cycles of this asymmetric swelling, buckling, and evaporative drying.



electrical energy. Ma *et al.* also showed that the mechanical energy from the water gradient could be converted to electrical energy and stored by incorporating a piezoelectric polymer on top of the composite film. Continuous mechanical actuation of the composite film produced alternating voltage pulses, which were rectified and stored in a capacitor.

The work of Ma *et al.* is important in that the smart combination of different materials substantially improved the overall device performance. Relative to the previous single-material system (6), the water-induced stress and water-induced strain of this composite system were greater by four orders of magnitude and by a factor of 6, respectively. The amount of mechanical power density is on the order of 1 W/kg.

Although it is interesting that the energy from the ambient moisture gradient can be harvested, many challenges must be met

to make the transition from the laboratory to successful commercial technology. One advantage of ambient energy harvesting is that the energy can be generated for a long period of time. The prototype form of Ma *et al.* is a polymer film, and power generation relies on mechanical distortion of polymer film. Polymer fracture may lower the device performance after many cycles of actuation. Also, there is a large gap between mechanical power density and electrical power density; the absolute electrical energy generated from the prototype is so low that its usefulness may be limited. However, it should be possible to increase the power output in a straightforward way by stacking multiple devices.

Although much work remains to be done, the power generator developed by Ma *et al.* can operate at low temperatures and does not require light, heat, or a temperature gradient. The composite actuator continuously moves by itself given a moisture gradient, which

can be readily found in natural environments. Although other ambient energy sources such as solar cells and thermoelectric generators have relatively high energy conversion efficiency and mechanical stability, energy harvesting from a water gradient may find applications in powering device networks.

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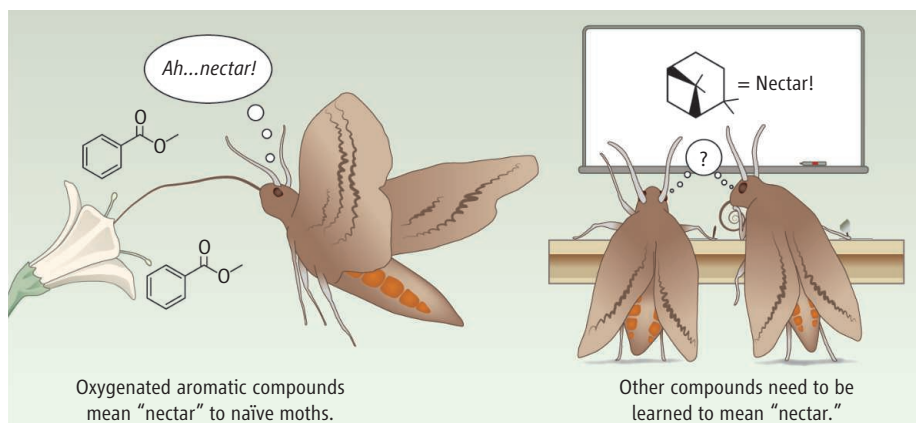
NEUROSCIENCE

Specialized But Flexible

Markus Knaden and Bill S. Hansson

Who can resist the bouquet of Christmas baking? It directly reminds us of positive experiences during early childhood. Odorants included in this mixture are also very likely to be innately attractive to humans, because they signal sugar and proteins. In contrast, most people can easily resist the flavor of surströmming, a Swedish national dish consisting of fermented herring that is—and smells like—rotten fish. Rotten fish emits compounds such as dimethyl sulfide, which is innately repellent to humans. The love of this food and the corresponding odors needs considerable learning. On page 200 of this issue, Riffell *et al.* (1) show that moths, which are usually specialized pollinators, can also learn to appreciate odors that are not their innate preference (see the figure). They also describe a potential neuronal substrate of this environmentally driven switch from specialized to flexible.

Whether an odor's valence is innate or has to be learned and how such processes are represented in the central nervous system are complicated questions to answer in humans,



Innate and learned odor preferences. Hawk moths have an innate preference for odors emitted by specific flowers. Riffell *et al.* now show that these moths can also learn to recognize and follow other odors. The authors are able to pinpoint the neuronal pathways that underlie these distinct preferences.

given the varied experiences we all have with different kinds of odors. The impact of innate and learned odor-directed behavior is far-reaching. For most animals, including humans, olfaction is the primary tool to estimate the value of a food source. Therefore, any changes of an odorant's valence might directly affect an animal's food preferences, and by doing so, its ecological niche.

Riffell *et al.* now report how the hawk moth *Manduca sexta* manages to locate

suitable food sources in a complex and changing environment. Ever since Darwin, it has been clear that the pollination-driven coevolution of flowers and moths led to extremely long corolla tubes on the plant's side and a long proboscis on the moth's side. Riffell *et al.* now add another layer to the mosaic of coevolution. They show that flower blends of plants that are mainly pollinated by hawk moths contain a characteristic mixture of oxygenated aromatic com-

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pounds. These compounds make the flowers smell completely different from flowers that attract other pollinators, such as bees. *M. sexta* seems to have evolved a strong innate preference for these key odors. By restricting their odor-based food search to odor plumes containing these key compounds, moths can save considerable time and energy, because other plumes could mislead them to less rewarding flowers (such as flowers that reward not with nectar but with pollen, a food source that cannot be used by most moths). Hence, specialization can be of great advantage.

However, the world is not usually that simple. To be equipped with an innate flower-odor preference is fine, as long as your habitat constantly provides you with the right type of flowers. But a hawk moth might enter a new habitat where none of its beloved flowers are blooming, or its current habitat might become depleted of the right type of flower. In such a situation, being too specialized would be lethal. Riffell *et al.* show that hungry moths are highly flexible and learn to follow plumes devoid of the above-mentioned cocktail of key aromatic compounds. The scientists were able to train the moths to be highly attracted to the flowers of *Agave palmeri*, a plant that is usually pollinated by bats and is not innately attractive to moths. But although the moths learned to nectar-feed on *Agave*, their preference for the originally attractive flowers was not affected. Given that the two behaviors seem to be independent of each other, the authors argue that learned and innate olfactory preferences might be processed along separate brain channels.

To understand how flower odors are processed, Riffell *et al.* performed electrophysiological recordings in the antennal lobe, the first olfactory processing center of the moth's brain. They found that output neurons of a specific region of the antennal lobe became strongly activated only by the bouquet emitted by flowers that contained the aromatic key compounds and, hence, were innately attractive. These neurons are equivalent to mitral cells in vertebrates that transfer information to higher brain centers.

A similar correlate between the innate valence of an odorant and coding in specific output neurons of the antennal lobe has also been shown in vinegar flies (2). However, by recording the neuronal activity patterns of moths while these moths were trained to innately unattractive *Agave* odors, Riffell *et al.* reached the next level. They showed that not only the innate valence but also the learned valence of an odor is processed at a

very early stage in the insect brain; the activation of those neurons that become activated by the emissions of the innately unattractive *Agave* rose significantly after moths had learned to forage on these flowers. The authors thus found a neuronal correlate in the brain to an environmentally driven modulation of behavior.

The physiological method used by Riffell *et al.*, who poked a multichannel electrode into the moth brain's region of interest, is probably not suitable to target the Swedes' strange preference for rotten fish. However, functional magnetic resonance imaging studies have shown that pleasant and

unpleasant odors are represented differentially in the orbitofrontal cortex of the human brain (3). It would be interesting if one, by following Riffell's reasoning, could analyze the activation patterns in this human brain region, in a non-Swede before and after he discovers his love for surströmming.

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NEUROSCIENCE

Developmental Refining of Neuroglial Signaling?

Antje Grosche and Andreas Reichenbach

A change in glutamate receptor expression in adult glial cells advances the current model of how glia regulate neuronal activity.

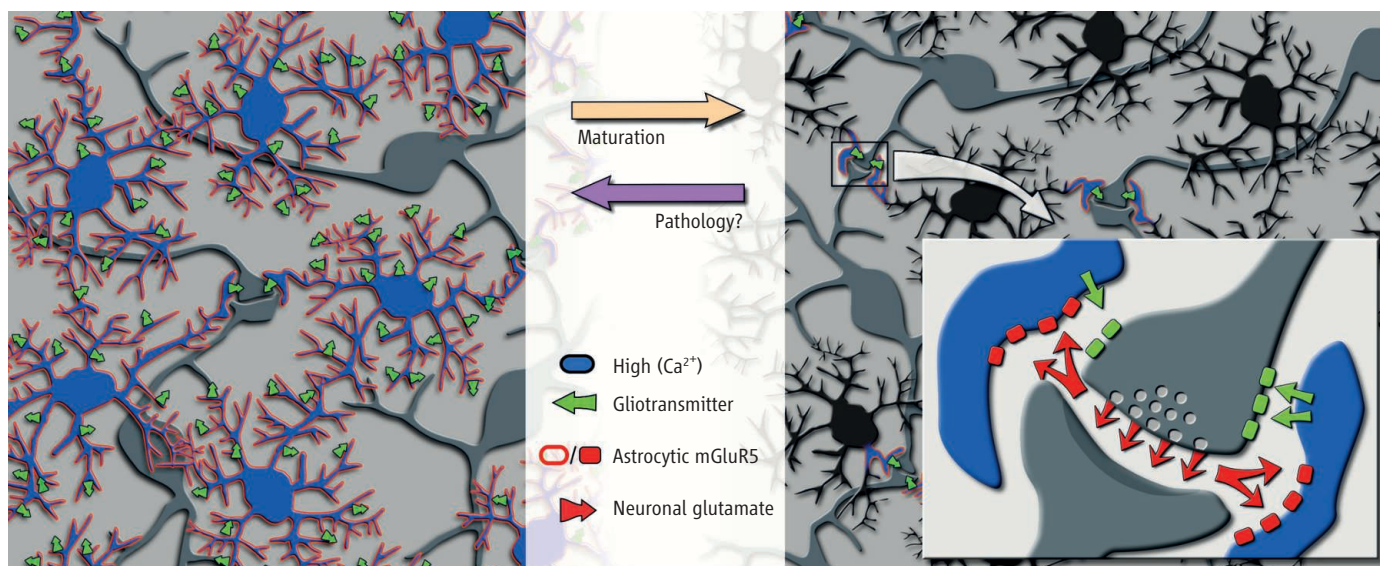
When the term neuroglia ("nerve-putty") was introduced by the German pathologist Rudolf Virchow in 1856 (1), it was like a stigma, distracting any reputable neuroscientist from doing research on these cells. More than a century later, intense research on glial cells was performed and culminated in the concept of the tripartite synapse, according to which astrocytes (a main type of glia) respond to neuronal activity by increasing their internal calcium (Ca^{2+}) concentration, which triggers the release of chemical transmitters from glia themselves and, in turn, causes feedback regulation of neuronal activity and synaptic strength (2). Although this concept became widely accepted among neuroscientists, it has now been challenged. On page 197 in this issue, Sun *et al.* (3) find that neuroglia signaling in the adult brain may occur in a manner fundamentally distinct from that exhibited during development.

The key finding of Sun *et al.* is that the expression pattern of metabotropic glutamate receptor subtypes (mGluRs) shifted during the second postnatal week in mice when the $\text{G}_{q/11}$ -coupled mGluR5 receptor was almost completely down-regulated in astrocytes,

whereas mGluR3 ($\text{G}_{i/o}$ -coupled) expression increased. Consequently, the intracellular signaling mechanism in response to extracellular glutamate changed from strong intracellular calcium rises (presumably eliciting gliotransmitter release in juvenile astrocytes) to a decrease in adenosine 3',5'-monophosphate (cAMP) concentrations (thereby lowering the probability of transmitter release in adult cells).

How does the tripartite synapse function in the developing and adult brain? Pioneering studies on gliotransmission found that $\text{G}_{q/11}$ -coupled receptor activation on glial cells caused an intracellular calcium rise, which was considered to be "necessary and sufficient" to initiate the release of gliotransmitters (4). Indeed, recent *in vivo* measurements in adult mice demonstrated a direct impact of glial calcium elevations (and subsequent glutamate or D-serine release) on the elicitation of long-term potentiation (5, 6). At cellular resolution, other studies showed that astrocytes can sense single synaptic events. In response to basal synaptic activity, rapid increases in calcium concentration in astrocytic microdomains (i.e., morpho-functional glial compartments interacting with a few synapses) (7) were detected (8, 9). These glial calcium rises appear to be involved in neuron-glia crosstalk. In the hippocampus, a gliotransmitter (probably ade-

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Tripartite synapses, maturing. A schematic hypothetical survey of mGluR5-mediated calcium (Ca^{2+}) rises and gliotransmitter release in multicellular glial networks during brain maturation (left) versus local responses in glial microdomains of the mature brain (right). These changes may be reversible after injury. Neurons are shown in gray.

nosine) is released from the activated astrocyte processes and enhances basal synaptic activity, thus providing a positive glial feedback mechanism (8). In the barrel cortex, astrocytic activity coincident with presynaptic stimulation evokes synaptic depression (9). Notably, the experiments on hippocampal astrocytes revealed the involvement of glial mGluR5 receptors (8), although they were performed in animals during the third postnatal week, when, according to Sun *et al.*, the mGluR expression has already shifted toward mGluR3 dominance. In summary, these data underline the relevance of changes in local astrocytic calcium concentrations for gliotransmission.

However, there are studies challenging this “calcium-centric” view. Some studies failed to detect alterations in the activity pattern of neuronal circuits after selective stimulation of astrocytic calcium rises via $\text{G}_{q/11}$ -dependent pathways (10). Moreover, as pointed out by Sun *et al.*, intercellular glial calcium waves were mostly reported in studies on slice preparations of young postnatal rodents. In vivo imaging techniques revealed that this phenomenon is absent in healthy adult brain (3, 11) but occurs in pathologically altered tissue (11). In the context of brain pathology, substances secreted by activated microglia (the immunocompetent cells in brain tissue) or by gliotically altered astrocytes (responding to tis-

sue damage might serve as upstream regulators of gliotransmitter release (12, 13). Indeed, enhanced gliotransmitter release is thought to occur as an early event in many, if not all, neurodegenerative diseases. Certainly, these exciting findings will stimulate future research on changes in gliotransmission, to identify potential glial targets for novel therapeutic approaches.

Taken together, the disparate results—obtained from different brain regions under a wide variety of conditions—imply that neuron-glia crosstalk is a very complex issue. Mature astrocytes have an intricate morphology, each forming intimate contacts with hundreds or thousands of synapses involving diverse neuronal circuits. Moreover, many glial membrane proteins other than mGluR5 may mediate neuron-to-glia signaling and glial calcium rises (14), and different neurotransmitters and glial receptors may be involved as well (5, 9). For the “classical” glutamatergic tripartite synapse, the reduction in mGluR5 expression during astrocyte differentiation described by Sun *et al.* coincides with a decrease in the spontaneous spindle burst activity of the neonatal brain (15) and thus presumably reflects a maturation process in neuron-glia signaling. Possibly, residual mGluR5 expression in astrocytic microdomains adjacent to glutamatergic synapses permits locally restricted, fine-tuned glial responses to individual synaptic activity while preventing widespread gliotransmitter release incommensurate with specific information processing (see the figure). The up-regulation of the $\text{G}_{i/o}$ -coupled mGluR3, eliciting cAMP decreases (3), may support this local restriction of glial responses. It remains to be seen whether strong astrocytic mGluR5

expression reoccurs during brain pathology.

The current state of the art prompts the hypothesis that neuroglial crosstalk at glutamatergic synapses becomes refined during functional brain maturation, with the glial Ca^{2+} rises becoming restricted from multicellular glial networks to discrete glial microdomains. This may contribute to the developmental shift from synchronous neuronal activity (required for establishment and maintenance of synaptic circuits) to specific information processing at individual synapses.

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INTRODUCTION

Inflammation's Yin-Yang

REDNESS, SWELLING, HEAT, PAIN. THESE WELL-KNOWN HALLMARKS OF INFLAMMATION were described over 2000 years ago by Celsus and are familiar to anyone who has gotten an infected cut or sprained their ankle. Fortunately, the symptoms of acute inflammation dissipate rapidly, and although inconvenient, they are also reassuring—they represent the immune system in action. The immune system clears the infection, guides the repair of damaged tissue, and the symptoms disappear.

Unfortunately, the very thing that is so beneficial during an infection can contribute substantially to the pathogenesis of several age-related chronic diseases such as metabolic disease/type 2 diabetes, cardiovascular disease, and neurodegenerative disease. In these diseases, saturated fatty acids, apolipoprotein B-containing lipoproteins, and the formation of protein aggregates, respectively, trigger activation of the immune system that results in inflammation. Thus, the immune system goes into overdrive. Because the stimulus that triggers the inflammation isn't easily cleared, it persists and contributes substantially to disease.

This special section in *Science* highlights the detrimental, but also in some cases beneficial, role of inflammation in neurodegenerative disease, cardiovascular disease, and metabolic syndrome. Aguzzi *et al.* (p. 156) focus on the role that microglia, resident macrophage-like cells in the brain, play in neurodegenerative diseases such as Alzheimer's disease, prion diseases, and traumatic brain injury. Although microglia can worsen disease, they are also important mediators of tissue repair and so also play protective roles in the brain. Swirski and Nahrendorf (p. 161) discuss the harmful and protective roles that leukocytes play in atherosclerosis and myocardial infarction. For instance, although leukocytes are important for tissue repair after a heart attack, their mobilization can worsen atherosclerotic plaques and lead to another infarction. Tabas and Glass (p. 166) use rheumatoid arthritis as a model to discuss how to develop therapies to target inflammation in cardiovascular disease. Finally, Odegaard and Chawla (p. 172) discuss how inflammation associated with obesity contributes to metabolic disease, but also point out the important role played by the immune system in maintaining an insulin-sensitive environment.

In *Science Signaling*, Research Resources by Warner *et al.* and auf dem Keller *et al.* highlight regulators of a receptor implicated in Crohn's disease (discussed in a Podcast with Gabriel Núñez) and the role of a matrix metalloproteinase in skin inflammation, respectively. A Perspective by Steinman discusses proinflammatory cytokines and diseases of the central nervous system, and a Perspective by Silke and Strasser focuses on the regulation of apoptosis and programmed necrosis *in vivo*.

These articles highlight the complicated roles that the immune system plays in chronic disease, suggest areas where more research and research tools are needed, and illuminate potential therapeutic strategies. Hopefully, careful manipulation of the immune system will add much-needed therapeutic tools to the arsenal to combat these already difficult-to-treat diseases.

— KRISTEN MUELLER

Inflammation

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Science

Microglia: Scapegoat, Saboteur, or Something Else?

Adriano Aguzzi,^{1*} Ben A. Barres,² Mariko L. Bennett^{2*}

Microglia are resident immune cells in the brain and spinal cord. These cells provide immune surveillance and are mobilized in response to disparate diseases and injuries. Although microglial activation is often considered neurotoxic, microglia are essential defenders against many neurodegenerative diseases. It also seems increasingly likely that microglial dysfunction can underlie certain neurological diseases without an obvious immune component.

The meaning of the term “inflammation” has undergone considerable evolution. Originally defined by Celsus’ four cardinal signs of “tumor, rubor, calor et dolor” (1), inflammation typically displays extravasation of blood cells—granulocytes in the beginning and lymphocytes soon thereafter. The word “neuroinflammation,” however, is increasingly used to identify a radically different set of conditions that are specific to the central nervous system (CNS). Whereas viral, bacterial, and autoimmune diseases of the CNS can resemble their extraneural counterparts morphologically, the concept of “neuroinflammation” has gradually expanded to also describe diseases that display none of Celsus’ cardinal signs, that do not attract conventional inflammatory cells, and that most neuropathologists would classify as degenerative rather than inflammatory. These “inflammatory” changes are restricted to a cell type exclusive to the CNS: the microglia.

Neuroinflammation is frequently viewed as deleterious to neurological function. Proliferation and activation of microglia occurs in most neurological diseases, from epilepsy to prion diseases. Consequently, the immune system is a major therapeutic target, even in CNS diseases whose primary pathogenesis is not obviously immunological. Indeed, the use of intravenously administered immunoglobulin to slow progression of Alzheimer’s disease (AD) is entering phase III clinical trials, and some patients with a fatal genetic demyelinating disease, X-linked adrenoleukodystrophy, now receive curative bone marrow transplants (BMTs) (2). Unfortunately, although there have been some victories, several high-profile immunotherapy trials for neurological diseases have recently failed (3). Few treatment options exist for patients, making the study of neuroimmune-mediated pathogenesis imperative.

In CNS inflammation, the blood-brain barrier (BBB) limits entry of patrolling bone marrow (BM)-derived immune cells. Instead, resident microglia perform normal surveillance. Microglia become rapidly activated in response to injury, undergoing morphological and molecular changes often associated with neurotoxicity. These changes are evident in postmortem human brain specimens, in animal models of disease, and more recently, with the use of positron emission tomography with radiolabeled benzodiazepine receptor ligands in human patients (4). Their proximity to pathology and an arsenal of potentially cytotoxic molecules make microglia a prime suspect in neurological disease. However, growing evidence about the origin and function of microglia underscores the complexity of this association and the importance of distinguishing microglia from BM-derived macrophages. Here, we highlight our current understanding of microglia in disease with an emphasis, where possible, on recent *in vivo* experimental data.

What Are Microglia?

Microglia are parenchymal tissue macrophages with delicate branching processes (“ramified,” or treelike) that include 10% of cells in the CNS. Unlike CNS macrophages found in the meninges, choroid plexus, and perivascular space, microglia derive from macrophages produced by primitive hematopoiesis in the yolk sac (5). These primitive macrophages migrate to the developing neural tube, where they give rise to microglia (6). BM-derived monocytes do not contribute to the mature microglial pool in the absence of conditioning irradiation to the CNS, suggesting that microglia are sustained by local progenitors (Fig. 1) (7, 8). Yolk sac–derived macrophages develop independently of Myb, a requisite transcription factor for stem cell development in the BM (9). Microglia and BM-derived macrophages thus represent two genetically distinct myeloid populations. These differences imply different functions for microglia and infiltrating macrophages, which are increasingly apparent in mouse models of disease (10, 11). Better methods are needed to specifically identify and manipulate each of these cell types.

Highly motile processes of “resting” microglia constantly survey the local microenvironment and rapidly respond to nearby injury with directed process or cell body migration (12, 13). Microglia clear apoptotic cells and are involved in both elimination and maintenance of synapses for proper neural circuit wiring. Some of these functions continue into adulthood. As our tools and technology for studying microglia grow, so too will our understanding of their role in the healthy brain (14).

Gene expression and morphological changes associated with microglial activation have been extensively studied (15). For example, neurons inhibit microglial activation through both receptor-ligand interactions that are dependent on contact (i.e., CD200 and its receptor) and secreted molecules such as fractalkine (CX3CL1) acting on the microglial receptor CX3CR1 [reviewed in (16)]. Derepression of these inhibitory influences likely promotes microglial activation. Recently, two proapoptotic caspases, caspase-8 and caspase-3/7, were shown to promote microglial activation downstream of the pattern recognition receptor Toll-like receptor 4 (TLR4) *in vitro* (17). In a chemical-lesion model of Parkinson’s disease (PD), pharmacological inhibition of caspase-3/7 suppressed the induction of proinflammatory mediators, such as inducible nitric oxide synthase and tumor necrosis factor- α , and provided modest protection against dopaminergic neuron loss. In postmortem brain specimens from AD and PD patients, caspase expression increases in activated microglia. Furthermore, signaling through estrogen receptor β (ER β) suppresses inflammatory responses of both microglia and astrocytes *in vitro* (18). Administration of ER β ligands prevented progression of experimental autoimmune encephalitis (EAE), a mouse model of multiple sclerosis. The induction of EAE has been attributed to microglia (19) as well as monocyte infiltration (11), making it difficult to ascribe this effect specifically to microglial responses.

Pumping Up Microglial Function to Treat AD

Accumulation of β -amyloid (A β) is a hallmark of AD, which contributes to the pathogenesis of both familial and sporadic AD (20). The net deposition of A β equals production minus clearance, meaning that the mechanisms of clearance are as important as those of A β generation. *In vitro* experiments strongly suggest a role for microglia in A β phagocytosis but are wrought with technical constraints common to existing microglia culture systems. First, the nonphysiological addition of serum to growth media provides opsonins and activating substances microglia do not normally see in the CNS. Second, many of these studies are hampered by the inability to isolate microglia from other macrophages.

Two recent studies suggest that microglia do not influence A β deposition or facilitate its clearance in multiple AD mouse models (10, 21).

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Selective depletion of microglia with the use of a lineage-ablation approach revealed no change in plaque formation, total A β load, or neurotoxicity in an AD mouse model (21). This finding establishes that although microglia accumulate around A β plaques, these cells do not contribute to their maintenance or clearance, at least on a short-term basis. To clarify a role for macrophages, BM chimeras were generated using lead to protect the brain and prevent peripheral monocyte infiltration (10). Selective loss of chemokine receptor 2 (CCR2) in perivascular macrophages decreased the ability of these cells to phagocytose A β and promoted an accumulation of A β

deposits surrounding the vasculature in another mouse model of AD. Restricted loss of CCR2 to this peripheral compartment resulted in cerebral amyloid angiopathy (22), supporting a role for perivascular macrophages in A β clearance from the CNS. But maybe these mouse models do not fully mirror the situation in humans, as polymorphisms in the microglial receptor TREM2 have emerged among the most potent risk modifiers for AD (23, 24).

Although microglia may be dispensable for A β clearance and disease progression in mice, can they be coaxed into helping to clear A β ? The strongest evidence for this concept comes

from the observation that A β plaques can be potentially cleared by antibodies against pyroglutamy-A β , an A β isoform that seems to exist only within plaques (25). Hence, A β can be cleared not only by creating an immunological sink in the periphery, but also by antibodies that selectively opsonize plaques.

Furthermore, polymorphisms of apolipoprotein E (apoE), a lipoprotein modulating phagocytic functions, are the most substantial genetic risk factor for sporadic AD. Expression of apoE is controlled by transcription factors—nuclear hormone receptors peroxisome proliferator-activated receptor gamma (PPAR γ) and liver X receptors (LXR)—whose long-term activation reverses neuropathological changes in A β -overexpressing mice (26). LXR and PPAR γ exert their function by forming heterodimers with retinoid X receptors (RXRs); treatment of A β mice with the RXR agonist bexarotene decreases A β levels and plaque burden and even restores cognitive functions (27), but only if mice express apoE. Though efficacy trials are needed to test off-label use of bexarotene (28), RXR agonism represents a promising therapeutic target for AD treatment.

Alternative Views of Repair

A key mechanism by which nuclear receptor activation promotes A β clearance may be the polarization, or differential activation patterns, of microglia. Macrophages, and probably microglia, are tuned for different types of host defense and protection, depending on the presence of available cytokines. Classical, or “M1,” polarizing signals arm macrophages to elicit proinflammatory and cytotoxic mediators, whereas alternatively activated “M2” macrophages can dampen inflammation and promote tissue regeneration (29). Although these two phenotypes probably fall on a continuum, PPARs act as master regulators of the M2 phenotype in macrophages. Pioglitazone, a PPAR γ agonist that confers protection in A β -overexpressing mice, strongly induces expression of M2-associated genes in microglia and macrophages (30).

Microglia and macrophage subsets may be beneficial in traumatic spinal cord injury (SCI). After SCI, peripheral monocytes infiltrate the injury site and differentiate into macrophages. These infiltrating cells are necessary for the limited functional recovery observed after SCI (31). Furthermore, both M1 and M2 macrophages are found at the injury site, with M2s constituting only a minority of cells. Interestingly, when isolated M2 macrophages are injected intraspinally after SCI, the expression level of M2-associated genes declines, suggesting that the injured CNS microenvironment suppresses the M2 phenotype (32).

Several findings suggest that proinflammatory macrophages are detrimental to CNS repair. Ablation of CX3CR1, which is expressed by microglia and some macrophages, promotes

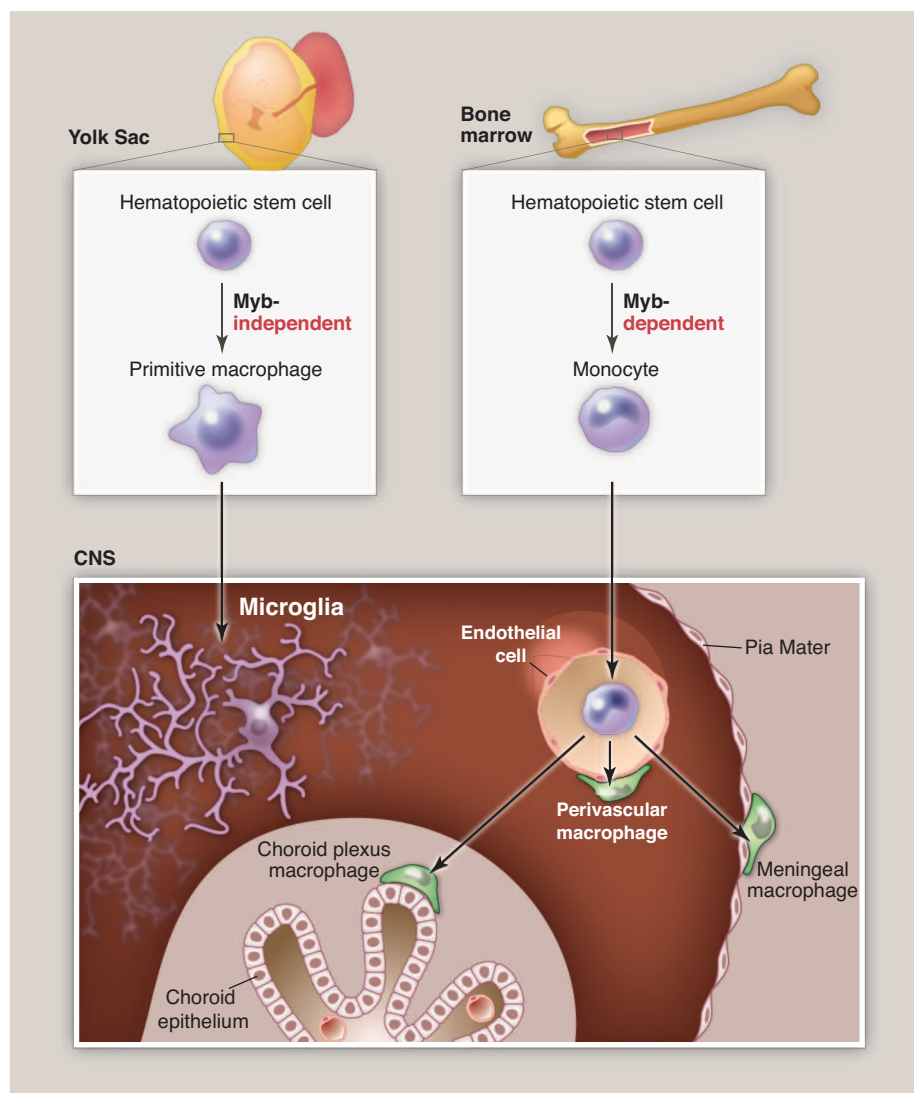


Fig. 1. Microglia derive from yolk-sac macrophages formed during primitive hematopoiesis beginning at embryonic day 7.5 (E7.5) (the embryo is depicted at E11.5 for illustrative purposes). Primitive macrophages migrate to the developing nervous system where they become microglia and reside throughout life. Local progenitors sustain the microglial population. In contrast, other CNS macrophages—found in the perivascular (Virchow-Robin) space, meninges, and choroid plexus—derive from precursor blood monocytes. Monocytes are formed in the BM from hematopoietic stem cells. Hematopoiesis in the BM is dependent on a transcription factor, Myb. However, this protein is dispensable for the formation of primitive macrophages.

Inflammation

functional recovery after SCI and results in smaller lesions (33). Peripheral loss of CX3CR1, which normally follows injury, prevents the infiltration of Ly6C^{lo}/iNOS⁺ (iNOS, inducible nitric oxide synthase) macrophages. It is unknown if the latter subset is M1-like, though *CX3CR1*^{-/-} mice exhibit decreased expression of proinflammatory cytokines in CNS macrophages and microglia after SCI. It is also possible that microglial loss of CX3CR1 contributes to CNS repair, perhaps by influencing the profile of infiltrating cells. Although functional recovery was limited, these data suggest that proinflammatory M1 macrophages are detrimental to repair.

Under certain circumstances, boosting inflammation can also promote CNS regeneration. Lens injury, or intraocular injection of TLR2 agonists, promotes axon regeneration after optic nerve injury, and combinatorial therapy with neuron-specific phosphatase and tensin homolog deletion and increased cyclic adenosine monophosphate facilitates long-distance axon regeneration and partial behavioral recovery (34). Finally, zymosan-activated macrophages stimulate axon regeneration—although when administered alone, they also trigger some neurotoxicity (35), highlighting the delicate balance of pro- and anti-inflammatory signals after CNS injury.

Although the above studies do not directly address the importance of microglial polarization in CNS repair, a study aimed at understanding the phenomenon of lipopolysaccharide (LPS)-induced neuroprotection may hold some clues (36). LPS is a TLR4 ligand that, when administered intraperitoneally, causes strong, proinflammatory microglial activation. Intrastriatal LPS injection is profoundly neurotoxic, yet repeated low-dose exposure to LPS affords neuroprotection against subsequent CNS injuries, an effect prevented by TLR4 ablation. The mechanism of preconditioning is intriguing because low-dose LPS treatment does not cause BBB breakdown or monocyte infiltration. Ablation of TLR4 from circulating immune cells did not suppress LPS-induced microglial activation and partial protection from cryogenic brain injury, whereas administration of wild-type (WT) BM to *TLR4*^{-/-} mice abolished those effects (36). Thus, expression of TLR4 by microglia, but not macrophages, is required for LPS-induced preconditioning. Finally, gene expression analyses of the whole brain after preconditioning reveal an increase in M2-associated transcripts, suggesting that LPS-conditioned microglia may be activated through an alternative pathway. Although the causality of M2 microglia in repair and protection needs further validation, these findings indicate that microglia are capable soldiers for CNS repair.

Microglia: Your Ally Against Prions

Although most brain diseases exhibit some degree of neuroinflammation, the most dramatic instance of microglial and astrocytic activation

is undoubtedly seen in prion disease. Progressive protein misfolding and aggregation of disease-associated prion protein (PrP^{Sc}) leads to fatal spongiform encephalopathies. At the terminal stage, the cortex of a patient with Creutzfeldt-Jakob disease often resembles a monoculture of excessively activated astrocytes and microglia, with very few remaining neurons (Fig. 2A). Microglia and macrophages can phagocytose PrP^{Sc} aggregates in vitro, but in vivo removal of PrP^{Sc} is relatively inefficient (37), and in the final stage of disease, prions ultimately win against microglia. Moreover, whereas phagocytosis is probably beneficial, failure to degrade prions may transform microglia into well-meaning yet lethal saboteurs that spread the disease by virtue of their motility.

To sort out the function of microglia in prion disease, cerebellar organotypic cultured slices from CD11b-HSVTK mice were treated with ganciclovir to induce microglial depletion (38). Surprisingly, loss of microglia resulted in vastly enhanced PrP^{Sc} deposits and augmented prion-infectivity titers. This effect was recapitulated in vivo with loss of milk fat globule-epidermal growth factor 8 protein (Mfge8), an astrocyte-secreted protein that binds phosphatidyl serine on the surface of apoptotic cells and promotes phagocytic clearance. Mfge8-deficient mice exhibited accelerated prion pathogenesis and increased numbers of apoptotic cell corpses, suggesting that Mfge8-mediated apoptotic cell clearance quells prion accumulation (Fig. 2B). Interestingly, the effect of Mfge8 depletion was visible in only

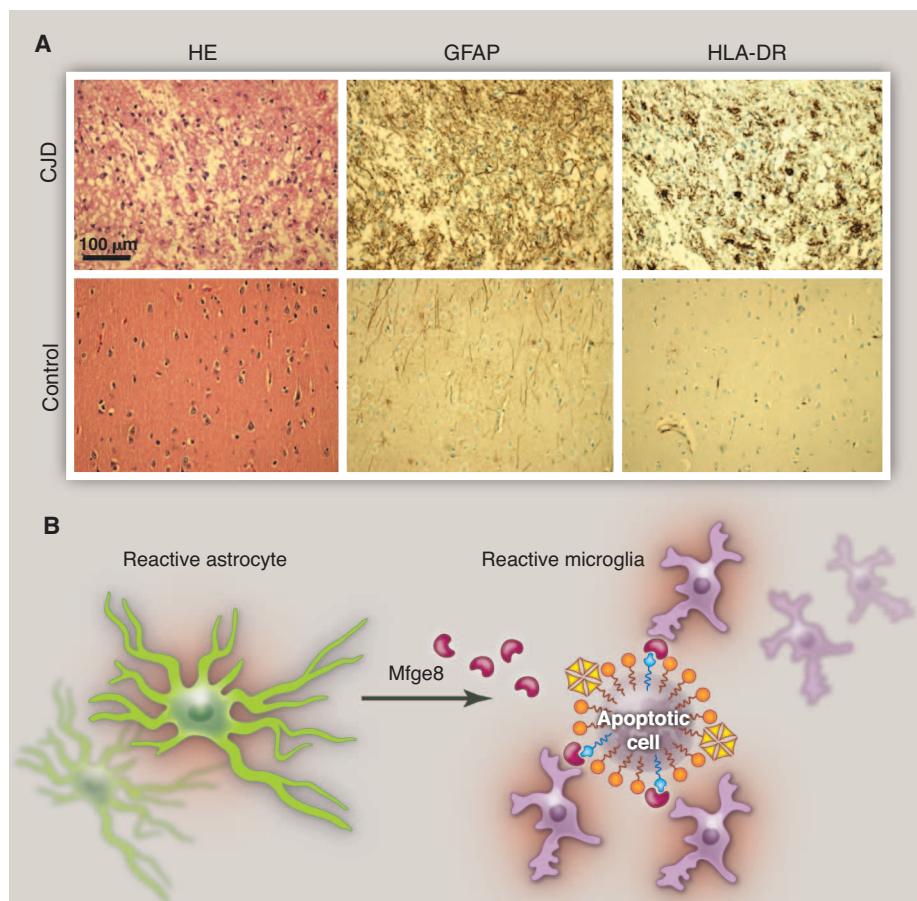


Fig. 2. Microglia and prions. (A) The cerebral cortex of a patient who died of Creutzfeldt-Jakob disease (CJD). The pyramidal cells are almost entirely obliterated and replaced by a monoculture of microglia and, to a lesser extent, astrocytes. Note the pseudolaminar pattern of vacuolation (“status spongiosus”), which indicates a ribbon of pathological prion deposition. HE, hematoxylin and eosin staining; GFAP, glial fibrillary acidic protein (an astrocyte marker); HLA-DR, human leukocyte antigen class II. **(B)** By virtue of its dual binding domains for both phosphatidyl serine and integrins, the secreted factor Mfge8 can bridge apoptotic cells and phagocytes (56). The excessive accumulation of PrP^{Sc} in Mfge8-deficient mice infected with prions suggests that microglia use the Mfge8 pathway to clear aggregated and misfolded proteins (38). Mfge8 is produced by astrocytes in the brain and by follicular dendritic cells in lymphoid organs, demonstrating that stromal cells secrete a local “licensing factor” that arms hematopoietic cells and enables the disposal of toxic detritus (57, 58).

certain mouse strains, implying the existence of further polymorphic determinants of prion removal, potentially including the Del-1 immunoregulator (39). Although astrocytes may also contribute to phagocytosis of these cells, these experiments reveal a protective function for microglia in prion disease and potential insights about the cross talk between microglia and astrocytes in neuroinflammation.

Development Complements Neurodegeneration

Developmental elimination of inappropriate synapses is critical for the proper wiring of the CNS. This process is mediated by proteins in the classical complement cascade, a robust immune sig-

naling pathway that tags debris or pathogens for phagocytosis by immune cells (40). During development, complement proteins C1q and C3 become localized to synapses. Recently, microglia were identified as the phagocytes responsible for elimination of tagged synapses, via complement receptor 3 (CR3) signaling (Fig. 3, A and B) (41). Immuno-electron microscopy revealed the active phagocytosis of presynaptic elements by microglia in the healthy, developing lateral geniculate nucleus (LGN). Mice without CR3 or C3 cannot effectively prune synapses, as evidenced by a sustained deficit in segregation of eye-specific axonal projections to the LGN. Microglia likely sculpt neural circuits throughout the CNS, be-

cause they also contribute in the developing hippocampus (42).

If complement proteins tag synapses for elimination by microglia during development, could this underlie the synaptic loss observed in this and other neurodegenerative diseases (Fig. 3C)? Examination of human brain tissue reveals strong induction of the complement cascade in many neurodegenerative diseases (43). In a mouse model of glaucoma, one of the most common neurodegenerative diseases, complement pathway expression is up-regulated long before retinal ganglion cell death (40). Immunohistochemistry revealed that robust C1q immunoreactivity in the microglia of mutant mice

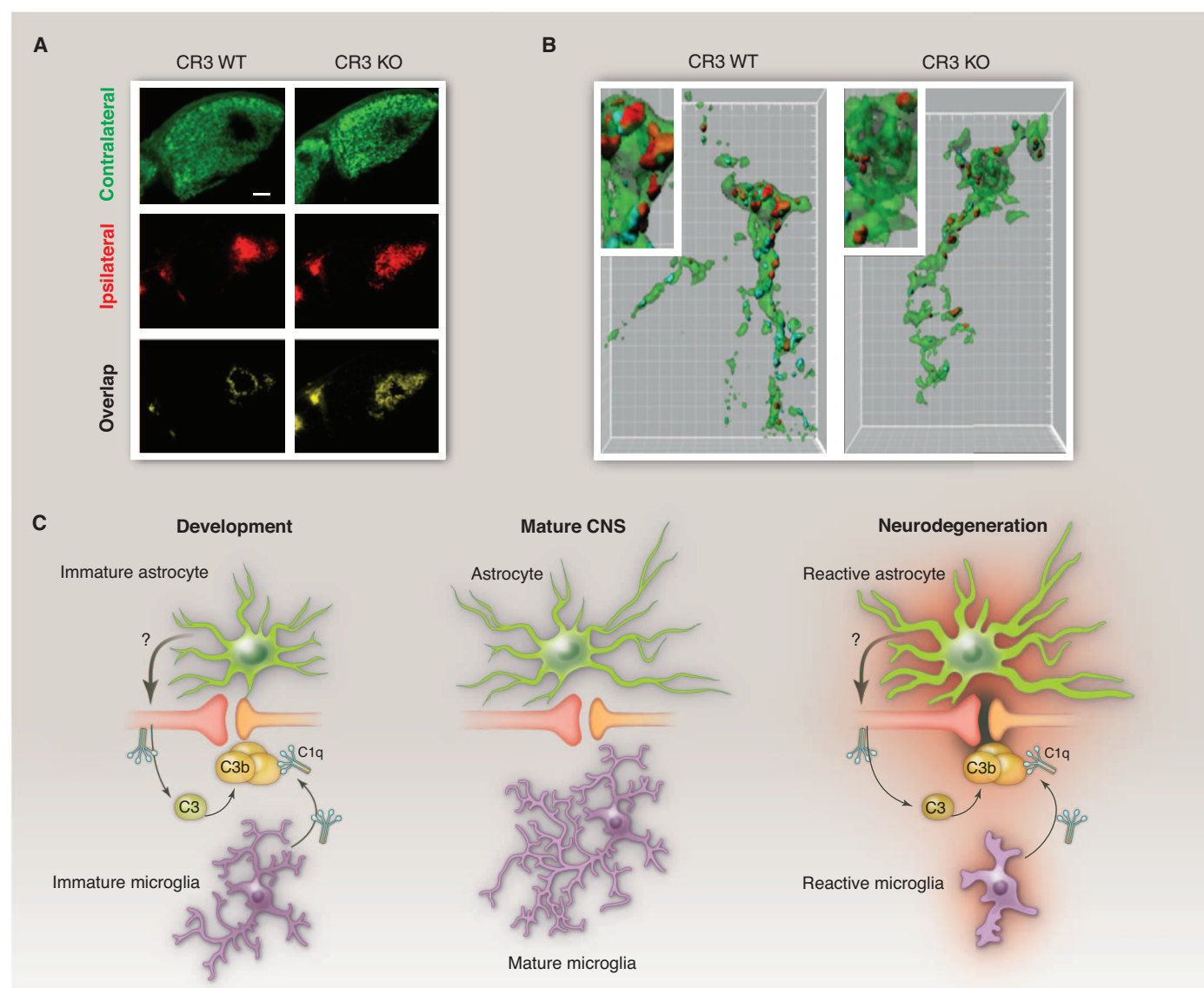


Fig. 3. Microglia prune synapses during development in a complement-dependent manner. Segregation of eye-specific inputs in the LGN requires the expression of CR3 (A) and C3. KO, knockout. Scale bar, 100 μ m. (B) Microglia in the developing LGN contain phagocytosed synaptic elements and cholera toxin B subunit-labeled axons from both the contralateral and ipsilateral eyes,

except in mice with deficient C3 signaling. [(A) and (B) are reproduced with permission from *Neuron* (41)] (C) This developmental process, in which an unknown factor released by astrocytes induces expression of C1q by neurons and microglia to promote C3-dependent synapse elimination, may be reactivated in neurodegenerative disease. [Adapted from (43)]

precedes pathological changes, suggesting that induction is an early event in pathogenesis (44). Importantly, loss of C1q strongly protected mutant mice from glaucoma.

Interestingly, microglial activation and synapse loss precede pathologic changes in mice carrying the Pro³⁰¹→Leu³⁰¹ human tau mutation (45) and raise the question of whether complement cascade inhibition could limit tauopathy in this model. Mice expressing a soluble variant of the natural complement inhibitor, complement receptor-related gene/protein y (sCrry) had decreased tau aggregation. In addition, inducing abnormal tau expression in mice lacking Cd59a, a potent inhibitor of the terminal stages of the complement cascade, resulted in increased tau aggregation and neuron loss compared with mice expressing the inhibitor (46).

Thus, complement cascade suppression is neuroprotective. The relation between complement induction and synapse loss in neuropathology is not yet clear. Neurodevelopment suggests that neural activity and signaling from astrocytes may induce complement expression (40). Are synapses in neurodegenerative disease inappropriately tagged, or are they inefficiently remodeled by aging or sick microglia? Understanding these mechanisms will likely reveal novel therapeutic strategies and clarify the function of microglia in neurodegenerative disease.

Microglia and Psychiatric Disease

Because microglia sculpt neural circuits, can dysfunction contribute to neurodevelopmental or psychiatric disorders? In an unexpected paradigm shift, immune cells may play a crucial role in diseases that are traditionally considered to be neuronal, including autism-spectrum and obsessive-compulsive disorders.

Pathological grooming in mice, reminiscent of the human impulse control disorder trichotillomania, is due to loss of Hoxb8 in the hematopoietic compartment (47). Mice with complete loss or specific deletion of Hoxb8 in Tie2-expressing cells (myeloid and endothelial cells) spent twice as long grooming as their littermates, leading to hair loss and skin lesions. Lethal irradiation of *Hoxb8*^{-/-} mice followed by BM reconstitution with WT cells rescues this phenotype. Donor BM from animals without B or T cells successfully restored normal grooming in *Hoxb8*^{-/-} mice, suggesting that expression of Hoxb8 by myeloid cells is compulsory for normal grooming. As a result of the strategy used for transplantation, donor cells engrafted into recipient brains, making it difficult to discern if Hoxb8 is required in microglia, macrophages, or both.

Microglia may also have an impact on Rett's syndrome, an X-linked autism spectrum disorder caused by the loss of function of methyl-CpG binding protein 2 (MeCP2). MeCP2 selectively binds methylated DNA (48) and controls the expression of epigenetically "imprinted" genes.

Girls affected by Rett's syndrome can develop normally during the first 6 to 18 months of life before developmental regression and the onset of seizures, irregular breathing, and progressive neurological signs. The connection between MeCP2 loss-of-function mutations and Rett's syndrome suggests that control of imprinting is crucial to neuronal function.

In male mice, *MeCP2*^{-ly} causes growth retardation, tremor, apnea, and gait and locomotor abnormalities. Loss of MeCP2 in neurons recapitulates the full knockout phenotype. Intriguingly, selective return of MeCP2 function to either microglia or astrocytes arrests Rett's syndrome pathology (49, 50). Surprisingly, restoration of MeCP2 expression in astrocytes of juvenile MeCP2-deficient mice prolonged life span, repaired respiratory function, and reversed neuronal abnormalities (50). Given the non-cell-autonomous effects of MeCP2, WT BM transplanted into MeCP2-deficient mice led to a dramatic improvement in life span, breathing patterns, and locomotor activity compared with mice transplanted with MeCP2-deficient BM (49). Preventing the engraftment of donor cells into the CNS by head-shielding during irradiation eliminated the benefits of MeCP2 reconstitution, suggesting that MeCP2⁺ microglia are required for the effect. The mechanism is unclear but may relate to phagocytic ability, because infusion of annexin V to prevent recognition of debris by phagocytes prevents the therapeutic effects of MeCP2 reconstitution. Thus, MeCP2's CNS function may be related to transcription of secreted factors necessary for normal neuronal development or redundancy of MeCP2-mediated functions by microglia, astrocytes, and neurons.

The findings with Hoxb8 and MeCP2 highlight an emerging principle in treating neurological diseases: If microglial dysfunction underlies a specific pathology, BMT may be an effective therapy. This theory has been successfully pioneered in X-linked adrenoleukodystrophy (2) and is being tested as a last-resort treatment for aggressive multiple sclerosis (51). Importantly, both of these conditions are associated with autoimmune infiltration of lymphocytes. Transplantation-based treatments for other neurological diseases, though exciting, warrant caution given the high risks of BMT. In addition, the emerging discord between microglial and BM-derived macrophage function necessitates future studies to determine whether engrafted BM-derived donor cells function like microglia.

Scapegoat or Saboteur?

One may be forgiven for concluding that our understanding of microglial biology is lacking, despite decades of research in this area (52). Yet, compelling experimental and clinical evidence exists in support of the notion that microglia act as both as "bad cop" and "good cop" in distinct circumstances.

Trying to identify the best microglial response to injury is difficult. The relationship of M1- and M2-polarized microglia is complex, because M1s might promote axon regeneration but be neurotoxic, and M2s may lack the necessary environmental signals to maintain their phenotype. At least in AD mouse models, increasing the M2 phenotype is promising, but the jury is still out for other diseases. Furthermore, overactivation or activation status may not be the only way in which microglia contribute to disease. Disruption of normal homeostatic functions of microglia lead to neural circuitry abnormalities, which could contribute to conditions lacking overt inflammation, such as neuropsychiatric disease (53).

It seems likely that our understanding of microglial function in health and disease will continue to diverge from that of other CNS and infiltrating macrophages. Such investigations require the development of new tools to manipulate microglia. Finally, whereas microglia are the resident myeloid cells of the CNS, astrocytes become reactive after injury, promote glial scar formation, are important components of neuroinflammation (54), and may function as phagocytes (55). The interplay between microglia and astrocytes is exciting and largely unexplored territory for inquiry. The findings in relation to Mfge8-mediated microglial phagocytosis of prion protein (38) and the as-yet-unidentified astrocyte signal that induced complement deposition at synapses are just the beginning (40). The function of astrocytes in disease and their cross talk with microglia may hold important clues about neurological pathogenesis.

Summary

Although the CNS was historically considered to be immune-privileged, the emerging presence of microglia in health and disease demonstrates that the CNS and the immune system are tightly coupled. Although CNS inflammation may have some differences from other tissues, it is every bit as nuanced. Studies investigating the contribution of microglia to disease processes identify beneficial, detrimental, and dispensable functions for these cells. However, new tools to specifically study microglial function as distinct from that of macrophages are now crucial to provide new insight into neuroinflammation and pathogenesis.

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REVIEW

Leukocyte Behavior in Atherosclerosis, Myocardial Infarction, and Heart Failure

Filip K. Swirski* and Matthias Nahrendorf*

Cardiovascular diseases claim more lives worldwide than any other. Etiologically, the dominant trajectory involves atherosclerosis, a chronic inflammatory process of lipid-rich lesion growth in the vascular wall that can cause life-threatening myocardial infarction (MI). Those who survive MI can develop congestive heart failure, a chronic condition of inadequate pump activity that is frequently fatal. Leukocytes (white blood cells) are important participants at the various stages of cardiovascular disease progression and complication. This Review will discuss leukocyte function in atherosclerosis, MI, and heart failure.

The immune system protects us against pathogens such as viruses, bacteria, and parasitic worms, but its influence is much broader. The system recognizes and responds to divergent environmental and endogenous stimuli, and every known disease is at least partially associated with or dependent on immune function. Cardiovascular disease is no exception.

Over the past half century, advances in public health (emphasis on healthy diet, exercise, and smoking cessation), clinical cardiology (chest pain units, coronary stenting, and cardiac defibrillation), and scientific discoveries (lipid-lowering statins, angiotensin-converting-enzyme inhibitors) have contributed to a steady decline in deaths from car-

diovascular disease (1). Despite these improvements, the disease is still responsible for 30% of deaths worldwide, surpassing all others, including cancer, and costing the global economy (in 2010) an estimated U.S. \$863 billion. Even after surviving myocardial infarction (MI) or stroke, the likelihood of developing secondary complications, such as reinfarction or heart failure, is high, which increases costs through hospitalizations and follow-up clinical care. The world population is rising, and it is estimated that by 2030 cardiovascular disease costs could increase to U.S. \$1,044 billion (2). Aside from addressing unmet medical needs at the public health policy and clinical care levels, a deep and nuanced understanding of the underlying biological processes should enable development of safe and effective treatments.

Atherosclerosis is the pathology that leads to MI and stroke. For many years after its recognition, atherosclerosis was thought to involve passive lipid deposition in the vessel wall. Today we

understand that atherosclerosis is a chronic inflammatory disease driven by lipids, specifically low-density lipoproteins (LDLs) and leukocytes. Neither atherosclerosis nor its complications adhere to a simple arithmetic of dietary lipid imbalance but rather comprise a syndrome in which environmental and genetic inputs disrupt biological systems. In other words, lifestyle, age, hereditary factors, and comorbidities disturb immune, digestive, endocrine, circulatory, and nervous systems, thereby altering immune function, metabolism, and many other processes while eliciting inflammation, hypercholesterolemia, and hypertension. Atherosclerosis develops and causes MI or stroke when many things go wrong in many different ways.

Leukocytes are keepers of the immune system. The various classes of myeloid and lymphoid cells that encompass the leukocyte repertoire recognize and eliminate pathogens and molecular patterns perceived to be dangerous. Working together, leukocytes can engender protective immunity and keep the host from harm. Yet they can also contribute to disease. Virtually every leukocyte class has been implicated in atherosclerosis and its complications, and their action is neither uniform nor hierarchical. Some leukocytes are atherogenic, whereas others are atheroprotective; some sustain inflammation after MI, yet others resolve it. This functional heterogeneity is a challenge but also an opportunity for therapeutic intervention because it suggests that specific disease-promoting functions can be targeted and those required for normal homeostasis can be spared.

Leukocytes in Atherosclerosis

The natural progression of atherosclerosis in the human involves the acquisition of specific features in the growing lesion. A key initiating process of atherosclerosis is the intimal retention of

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Inflammation

apolipoprotein (apo) B-containing lipoproteins in regions of disturbed blood flow and low shear stress (3). Lipid-rich macrophages, otherwise known as foam cells, appear early in the intima and can be identified in nearly 40% of newborns; they typically regress before the age of 2. The existence of small pools of extracellular lipids in the intima is a feature of a preatheroma, whereas an easily discernible core of extracellular lipid marks an atheroma. Increasingly complicated lesions are defined by fibrous thickening; the appearance of fissures, hematoma, and thrombi; and calcification. By the age of 40, 95% of people have some type of lesion. Problems occur if a lesion interferes with tissue oxygenation when either the lesion's size reduces blood flow or the lesion ruptures and occludes the vessel altogether. Of the two, lesion rupture is far more dangerous. MI and stroke are sudden events that result from occlusion of vessels that oxygenate the heart and brain, respectively (4).

Macrophage as protagonist. Macrophages are most numerous among leukocytes in any type of lesion and, with the possible exception of smooth muscle cells, the most prominent cellular contributors to the lesion's physical bulk (5). In response to intimal lipid accumulation, disturbed blood flow, low shear stress, and other stimuli, endothelial cells permit monocytes (major precursors of macrophages) passage across the endothelium (6, 7). Newly infiltrated monocyte-derived macrophages recognize and ingest lipids that have accrued in the intima as a consequence of hypercholesterolemia. Macrophages are specialized phagocytes that rely on different strategies to sense, internalize, and process the diverse lipid moieties they encounter. Pattern recognition receptors (PRRs) expressed on the plasma membrane

recognize various native and oxidized lipoproteins and facilitate their uptake for lysosomal degradation (8). Likewise, cytoplasmic sensors such as the NLRP3 inflammasomes respond to cholesterol crystals and release interleukin-1 β (IL-1 β), a major inflammatory cytokine (9, 10). The relative importance of specific sensors to the generation of foam cells and development of atherosclerosis continues to be debated: PRRs such as SR-A and CD36 are dispensable to foam-cell formation (11), whereas the NLRP3 inflammasome influences atherosclerosis in one mouse model (9) but not another (12).

Macrophage lipid uptake may be viewed as a protective response that backfires. Ingesting oxidized lipoproteins, like ingesting microbes, involves the scavenging of substances perceived to be dangerous. After lipoprotein recognition and consequent ingestion, macrophages morph into foam cells, many of which eventually die and contribute to a large lipid core, a characteristic of lesions most vulnerable to rupture (5). Surprisingly, foam-cell formation suppresses, rather than promotes, inflammatory gene expression (13). Leukocytes may fuel an inflammatory cycle, but they also, in some incarnations, quench it.

Hypothetically, macrophages can prevent atherosclerosis by scavenging and removing surplus lipoproteins from the artery wall, without becoming foam cells and without escalating inflammation. Can such a scenario exist? Several lines of investigation on the production, migration, and function of macrophages provide clues.

Leukocytosis predicts cardiovascular events. Human and mouse blood contains at least two distinct monocyte subsets with differential migratory properties (14). Mouse Ly-6C^{high} monocytes

share several properties with human CD16⁺ CD14⁺ monocytes and are inflammatory. In response to hypercholesterolemia, the bone marrow and spleen overproduce Ly-6C^{high} monocytes that enter the circulation, contribute to excessive monocytosis, preferentially accumulate in lesions, and differentiate to macrophages (15–17) (Fig. 1). Ly-6C^{low} monocytes, which are thought to be primarily reparative, are similar to CD16⁺ CD14^{dim} human monocytes, patrol the vasculature, and infiltrate atheromata less frequently. If an overzealous immune system generates too many cells that contribute to pathology, then targeting the culprit subset may be atheroprotective.

Genetically engineered disruption of cholesterol metabolism defines the two major models of atherosclerosis: Apolipoprotein E-deficient (*Apoe*^{−/−}) and LDL receptor (LDLR)-deficient mice develop atherosclerotic lesions with striking resemblance to those that evolve in people. Between the two, *Apoe*^{−/−} mice develop more severe blood leukocytosis and monocytosis. Recent studies, using *Apoe*^{−/−} and other mice deficient in lipid-efflux proteins such as the adenosine triphosphate (ATP)-binding cassette transporter 1 (ABCA1) and ATP-binding cassette subfamily G member 1 (ABCG1), have forged a mechanistic link between leukocyte and lipid biology. Trapped cholesterol in hematopoietic stem and progenitor cells (HSPCs) that lack the crucial cholesterol efflux machinery leads to the expression of the granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-3 common beta chain receptor on the plasma membrane and contributes to excessive proliferation (18, 19). In other words, cholesterol efflux suppresses proliferation. Concurrently, lipid-rich splenic phagocytes release IL-23,

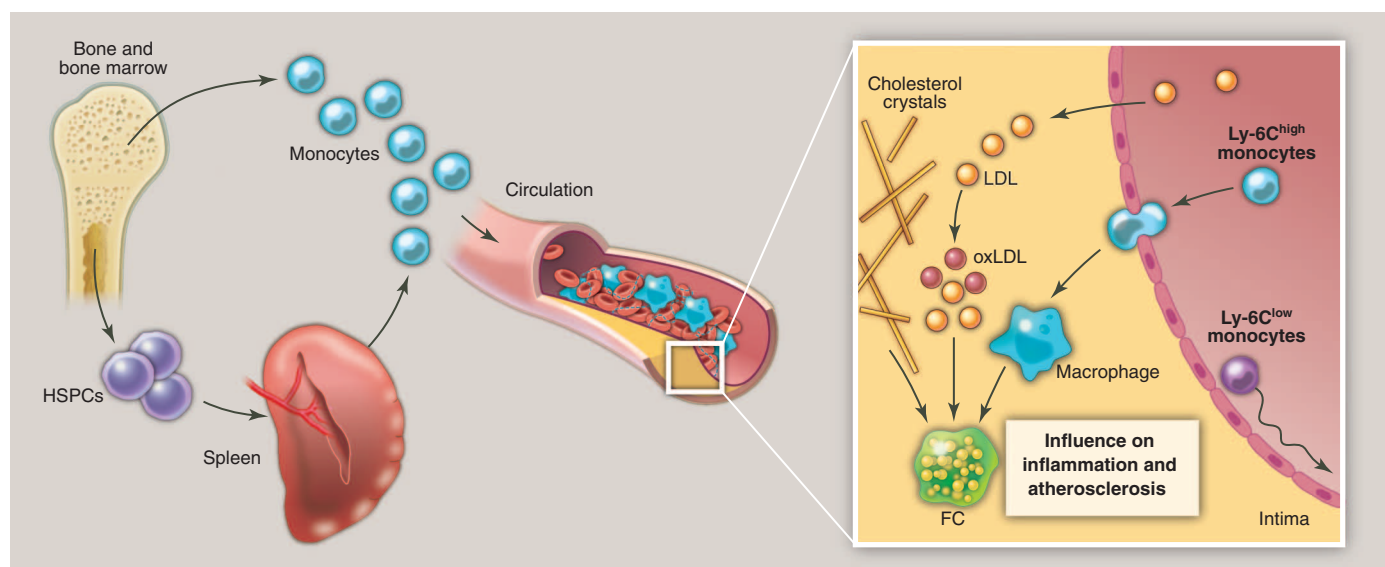


Fig. 1. Monocyte flux and function in experimental atherosclerosis. Monocytes are made in bone marrow (medullary hematopoiesis) and spleen (extramedullary hematopoiesis). Ly-6C^{high} monocytes accumulate preferentially in the lesion and differentiate to macrophages, which ingest lipids or cholesterol

crystals, and become lipid-rich foam cells (FC). Ly-6C^{low} monocytes patrol the vasculature and enter lesions less frequently. Concurrently, other leukocytes and platelets participate in inflammation and lesion growth in various consequential ways (see main text).

which induces a cascade that eventually liberates HSPCs from their medullary niches (20). When HSPCs seed extramedullary sites, they encounter GM-CSF and IL-3 (17). The net effect is HSPC proliferation, extramedullary hematopoiesis, leukocytosis, and accelerated atherosclerosis.

Most humans are not apoE, LDLR, ABCA1, or ABCG1 deficient; they are not impaired in handling cholesterol. But human cardiovascular disease does associate with leukocytosis (21). In comparison with the mouse model in which a defect in cholesterol clearance leads to severe systemic inflammation, human leukocytosis is almost always mild. The mouse phenotype may represent an extreme version of a process that merely simmers in people, perhaps a version of insufficient cholesterol handling rather than its complete breakdown. If so, then precise targeting of leukocyte-centric cholesterol pathways will be therapeutically desirable.

Macrophage flux and function. Macrophage flux involves monocyte migration across the endothelium, monocyte differentiation to macrophages, macrophage retention in the atheromata, exit, or death (Fig. 1). The preferential accumulation of Ly-6C^{high} monocytes in the growing atheromata relies on chemokine-dependent signaling through CCR2-CCL2, CX3CR1-CX3CL1, and CCR5-CCL5 (16), and neutralizing these axes in mice almost abolishes atherosclerosis (22, 23). Upon accumulation and lipid ingestion, macrophages release netrin-1, a guidance molecule that binds to UNC5b on the plasma membrane and blocks the directed migration of macrophages out of the lesion (24). In the absence of netrin-1, lesions are smaller. The relative contribution of macrophage exit and/or death may indeed be critical to how disease progresses. On the one hand, efficient macrophage exit (25), or reduced monocyte influx (26), is central to regression; when lesions regress, macrophage numbers diminish. On the other hand, limited macrophage death, effective efferocytosis (phagocytosis of dead cells), and active autophagy are fundamental to lesional stability (27); lesions with large necrotic cores are most vulnerable.

A macrophage is not simply “a big eater,” however, and it does not exist in isolation. Lesional macrophages are polyfunctional and interact with other leukocytes and nonleukocyte client cells. Macrophage polyfunctionality in the aorta represents either dedicated behavior of individually specialized cells (distinct macrophage subsets) or adaptive behavior elicited by the tissue environment (macrophage plasticity). Regardless of how division of labor is maintained, macrophages, as a cell population that accumulates lipids, secrete cytokines that attract other leukocytes, produce proteinases that digest the extracellular matrix, disturb smooth-muscle cell function, and can influence endothelial-dependent vasodilatation (5). Macrophages can also resolve inflammation and promote granulation tissue formation

(28). Aside from, or perhaps in addition to, controlling LDL levels, a therapeutic goal for atherosclerosis may be to coax macrophage responses away from inflammation and proteolysis and toward robust cholesterol transport, intelligent cellular flux, and sustained efferocytosis.

Adaptive immunity in atherosclerosis. Is adaptive immunity—the immune system’s arm that uses antigen specificity to generate potent defense and lasting memory against specific pathogens—important in atherosclerosis? Dendritic cells, which capture an antigen and present it to naive T cells, reside in the aorta and affect progression of atherosclerosis (29–31), possibly by interacting with T cells (32) previously activated in lymph nodes and tertiary lymphoid organs (33). T cells are relatively rare in lesion. Even so, specific perturbations, mostly of T cell–associated cytokines, suggest that T helper (T_H)–1 and T_H17 cells are atherogenic, whereas T_H2 and regulatory T (T_{reg}) cells are protective (34, 35). T_H1-generated interferon (IFN)– γ , for example, activates macrophages and propagates inflammation, whereas T_{reg}-generated IL-10 and transforming growth factor (TGF)– β dampen inflammation. B cells, which are rare in lesions but more prevalent in the adventitia, can be both atheroprotective and atherogenic (36, 37). The innate like B1 B cells are atheroprotective, possibly because they produce natural immunoglobulin M (IgM) antibodies that mark lipids for Fc receptor–mediated removal. The adaptive B2 B cells, on the other hand, contribute to disease, presumably by interacting with other leukocytes and/or secreting inflammatory cytokines (36). Because cellular and molecular constituents implicated in adaptive immunity regulate atherosclerosis, they can be harnessed, at least experimentally, to alter disease progression.

The generation of antigen specificity through genetic recombination defines adaptive immunity. In atherosclerosis, antibodies and CD4⁺ T cells that react to lipid moieties such as oxLDL have been identified (38, 39). However, lymphocytes can secrete cytokines and proliferate in response to germ-line-encoded receptor signaling. Innate lymphoid cells, natural killer T cells (NKT cells), tissue-resident $\gamma\delta$ T cells, and innatelike B cells such as innate response activator (IRA) B cells share phenotypic features with their adaptive counterparts but differ in how they perceive and respond to molecular patterns. The relative importance of germ-line-encoded and genetically recombined receptors in recognizing relevant atherosclerotic antigens and influencing disease progression remains unknown.

Inflammatory professionals. Neutrophils, mast cells, and platelets are detectable in lesions and may influence atherosclerosis and its complications in important ways. Neutrophils, the inflammatory granulocytes, circulate in large numbers and accumulate early on at sites of injury or infection. In experimental atherosclerosis, neutrophils, whose numbers rise in the blood, help

monocyte adhesion or transmigration by releasing alarmins and other preformed granular proteins (40). Neutrophils also contain large quantities of myeloperoxidase, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, and lipoxigenases, which contribute to oxidative stress, a major determinant of endothelial cell dysfunction, lesion growth, and instability. Mast cells, best known for their role in allergy and anaphylaxis, promote atherosclerosis by releasing the contents of their protease-cytokine-autacoid-rich granules (41). Platelets play an essential, dual role. During atherosclerosis, they adhere to the endothelium and help monocytes enter lesions (42). During plaque rupture, they form the thrombus that causes ischemia of downstream tissue (5). Thus, neutrophils, mast cells, and platelets all promote atherosclerosis by intensifying inflammation.

Leukocytes in Myocardial Infarction and Heart Failure

Leukocytes as friends and foes of the heart. Small atherosclerotic lesions that do not hinder blood flow are found in many people. Subclinical atherosclerosis manifests itself when lesions reduce tissue oxygen supply. Adluminal lesion growth and inward arterial remodeling gradually narrow the vessel diameter, thereby limiting blood flow. If coronary arterial stenosis reaches more than 80%, downstream heart muscle becomes ischemic, especially when a high cardiac work load increases oxygen demand. Typically, myocardial ischemia leads to chest pain, which occurs during exertion (stable angina pectoris). Plaque rupture, which is precipitated by leukocyte action, suddenly occludes the artery and causes unstable angina and MI. In contrast to atherosclerosis, where chronic low-grade stimulation by native and oxidized lipoproteins mobilizes leukocytes to the vessel wall, in MI, acutely dying myocytes elicit different triggers that nevertheless recruit similar classes of leukocytes. In response to the MI, the leukocyte profile in the myocardium changes drastically. The population of macrophages (43) and dendritic cells (30) that reside in the healthy heart is quickly overwhelmed by inflammatory leukocytes.

Shortly after onset of ischemia, endothelial cells up-regulate adhesion molecules that, along with released chemokines, trigger neutrophil extravasation. Rapid accumulation leads to an early neutrophil peak after injury (28). In skin wounds, neutrophils protect against infection through phagocytosis of bacteria and release of reactive oxygen species. In the ischemic heart, neutrophils phagocytose dead tissue and release inflammatory mediators: an immunological misfire aimed at myocytes that survived the ischemic injury. It is believed that neutrophils are deleterious in MI, but they eventually undergo apoptosis and are removed by macrophages.

Aside from neutrophils, inflammatory Ly-6C^{high} monocytes are among the earliest responders. Distressed tissue expresses CCL2 (44), which

Inflammation

attracts Ly-6C^{high} monocytes during the first several days, after which the wounded heart switches to CX3CL1-mediated recruitment of Ly-6C^{low} monocytes (28). The cause of the switch is unknown, but sequentially accumulating monocyte subsets do pursue differential functions in the infarct (Fig. 2). Although both are phagocytic, the early inflammatory subset is particularly rich in proinflammatory mediators. These include proteases, which are tightly regulated to balance wound debridement with tissue destabilization, because unchecked proteolysis may cause infarct expansion or even rupture. During resolution of inflammation, reparative Ly-6C^{low} monocytes support angiogenesis and extracellular matrix synthesis by providing vascular endothelial growth factor (VEGF) and TGF- β (28). Whether Ly-6C^{high} and Ly-6C^{low} monocytes give rise to distinct macrophage subsets in the infarcted myocardium is unknown, but more intriguing is the possibility that monocytes, as monocytes, impose a lasting effect on the tissue environment before differentiating (or dying or exiting). Decades of research have argued that macrophages are highly susceptible to microenvironmental cues. Perhaps recruited monocytes influence a tissue in a manner that long-seeded, sessile macrophages no longer can.

With a short time delay and at lower numbers when compared to the infarct, monocytes also invade the nonischemic remote myocardium (45). Here, myeloid cells may contribute to various processes: They may cause heart failure by facilitating ventricular dilatation via proteolysis of the colla-

gen matrix that lends mechanical stability to the heart; they may activate fibroblasts and promote interstitial myocardial fibrosis; they may harm myocytes through secretion of proapoptotic factors. But myeloid cells could also protect the non-ischemic remote myocardium, for instance through secretion of angiogenesis-promoting cytokines. The function of myeloid leukocytes in this region has yet to be determined.

Lymphocytes are present in low numbers in the infarct and proliferate in draining lymph nodes shortly after ischemic injury (46). CD4⁺ T cell deficiency delays the transition from Ly-6C^{high} to Ly-6C^{low} monocyte presence and impairs healing of the heart. Likewise, depletion of dendritic cells disturbs resolution of inflammation (47). These observations suggest a link to adaptive immunity, but it is unclear, given that MI is a form of sterile injury, whether any processed peptide antigens are recognized and presented. MI may be mobilizing elements of adaptive immunity without necessarily relying on its essential mechanisms.

The emerging picture positions leukocytes as both protective and harmful in MI. Ly-6C^{high} monocytes, for example, are required during the initial response to ischemia but can also be destructive if they persist in the infarct too long. Ly-6C^{low} monocytes, on the other hand, are probably essential to wound repair (28, 48). Optimal healing requires balance: Sequential and coordinated cell recruitment that removes dead tissue and strengthens the wound so that the heart performs its vital function is a manifestation of that

balance (28); comorbidities can disrupt it (49). Therapeutic strategies should therefore aim to recalibrate the leukocyte response toward optimal healing. In vivo RNA interference is one strategy that can selectively silence CCR2 at defined time points, thus limiting the accumulation of Ly-6C^{high} monocytes in atherosclerotic plaques and the infarcted myocardium (50). As our understanding of how various leukocytes regulate atherosclerosis and its complications enlarges, so will the potential repertoire of targeted therapeutics.

Leukocytes anchor system-wide mayhem after MI. Acute MI is a severe traumatic event that mobilizes multiple organ systems (Fig. 3). A substantial number of the initially recruited monocytes derives from a reservoir in the splenic red pulp, where increased angiotensin-2 signaling triggers their mobilization within hours after injury (51). For the first few days after infarction, leukocyte recruitment remains at astonishing levels (48). To meet the demand, cell production increases in the bone marrow and the spleen. How do the myelopoietic sites learn about the need for cells in the heart? Pain and anxiety during acute MI alert the sympathetic nervous system and activate neuroimmune synapses (52). In the bone marrow, sympathetic nerves release noradrenaline, which binds to β_3 adrenergic receptors expressed by mesenchymal stem cells (MSCs). These cells are part of a housekeeping team that regulates leukocyte progenitor cell activity in the bone marrow niche. In response to noradrenaline, MSCs withdraw the HSPC

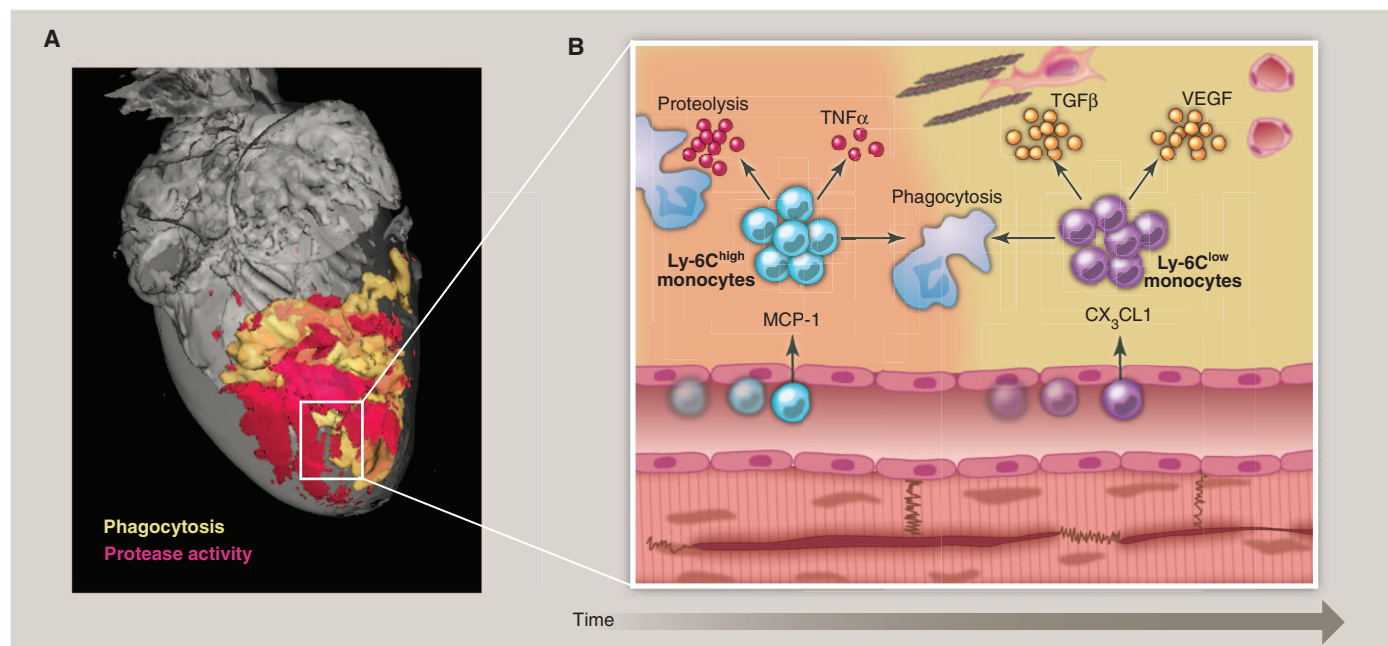


Fig. 2. Biphasic monocyte response during early myocardial remodeling. (A) Optical projection tomography of a murine heart with MI after injection of molecular imaging reporters for major function of myeloid cells, including protease activity (red) and phagocytic activity (yellow). [Image: Courtesy of C. Vinegoni] (B) Cartoon of biphasic monocyte subset activity in the

infarct as a function of time. In a first phase, inflammatory Ly-6C^{high} monocytes are recruited via monocyte chemoattractant protein-1 (MCP-1, also known as CCL2) and remove necrotic debris. In a second phase, Ly-6C^{low} monocytes accumulate via CX3CL1 and pursue repair functions that result in a stable scar.

retention factor CXCL12 and liberate HSPCs into circulation. These cells then seed the spleen and amplify extramedullary myelopoiesis, enabling the organ to contribute monocytes beyond its baseline reservoir function. Local environmental changes in the spleen, including increased levels of IL-1 β and stem cell factor (SCF/kit ligand), orchestrate the emergency cell production after MI (48, 52). Other still unknown soluble factors may act as alarmins to trigger emergency hematopoiesis after MI.

The next turn in a vicious cycle? Regardless of how many leukocytes are needed in the infarcted myocardium, the activated endothelium that lines (unruptured) atherosclerotic plaques can recruit leukocytes that have become available in increased numbers as a result of the MI. This may partly explain why atherosclerotic lesions grow faster and develop a more advanced phenotype shortly after ischemic injury (53). Because the inflammatory burst after MI generates proteolytic leukocytes, their “off-target” accumulation in atherosclerotic lesions may disrupt the fibrous cap, increasing the likelihood of reinfarction. Leukocyte activity post-MI should therefore be considered as a therapeutic target for secondary prevention.

Derailed infarct healing contributes to heart failure. MI is frequently studied in young mice subjected to coronary ligation. Young and healthy humans, however, rarely suffer from MI. Atherosclerosis, risk factors, and comorbidities such as diabetes and obesity—many of which have an inflammatory component (54)—typically precede ischemic injury. The smoldering systemic inflammation impedes infarct healing by interfering with the resolution of local inflammation and delaying the reparative phase (49). Consequently, the infarct expands and the left ventricle dilates, ultimately leading to heart failure. This frequent syndrome, defined by the inability to pump sufficient quantities of oxygenated blood into peripheral tissues, manifests with shortness of breath and fluid retention and carries a mortality as high as 50%. Data on leukocyte activity in the chronically failing myocardium are unavailable; however, inflammatory biomarkers, such as C-reactive protein, and inflammatory mediators, such as the cytokines tumor necrosis factor- α (TNF- α) and IL-6, increase systemically in heart failure, and leukocytosis associates with disease progression. Leukocytes are present in the myocardium of mice with chronic heart failure induced by pressure overload (55). Given leukocytes’ role in many

other disease settings, their function in patients with chronic heart failure should be examined.

Human data suggest that insights obtained in rodents can be translated to the clinic. Leukocytosis and inflammatory monocyte levels correlate with post-MI heart failure in patients (56, 57). After acute MI, the human bone marrow increases activity (58) and releases HSPCs into circulation (59), possibly resulting in splenic proliferation (52). Two monocyte subset peaks occur in the blood of patients with acute MI (57), suggesting that the infarcted human myocardium likewise mobilizes monocyte subsets in distinct phases. The similarities between human and rodent data are encouraging but must be viewed with caution given the many dissimilarities between human biology and rodent models of human disease. That being said, there is immense value in what animal models can reveal. The integration of knowledge from divergent disciplines—human genetics that searches for susceptibility loci by using gene-wide association studies, molecular biology that describes and forges links between intracellular metabolic and immune pathways, and cellular immunology that monitors leukocyte communication in murine organ systems—ought to be a focus of a concerted effort in cardiovascular

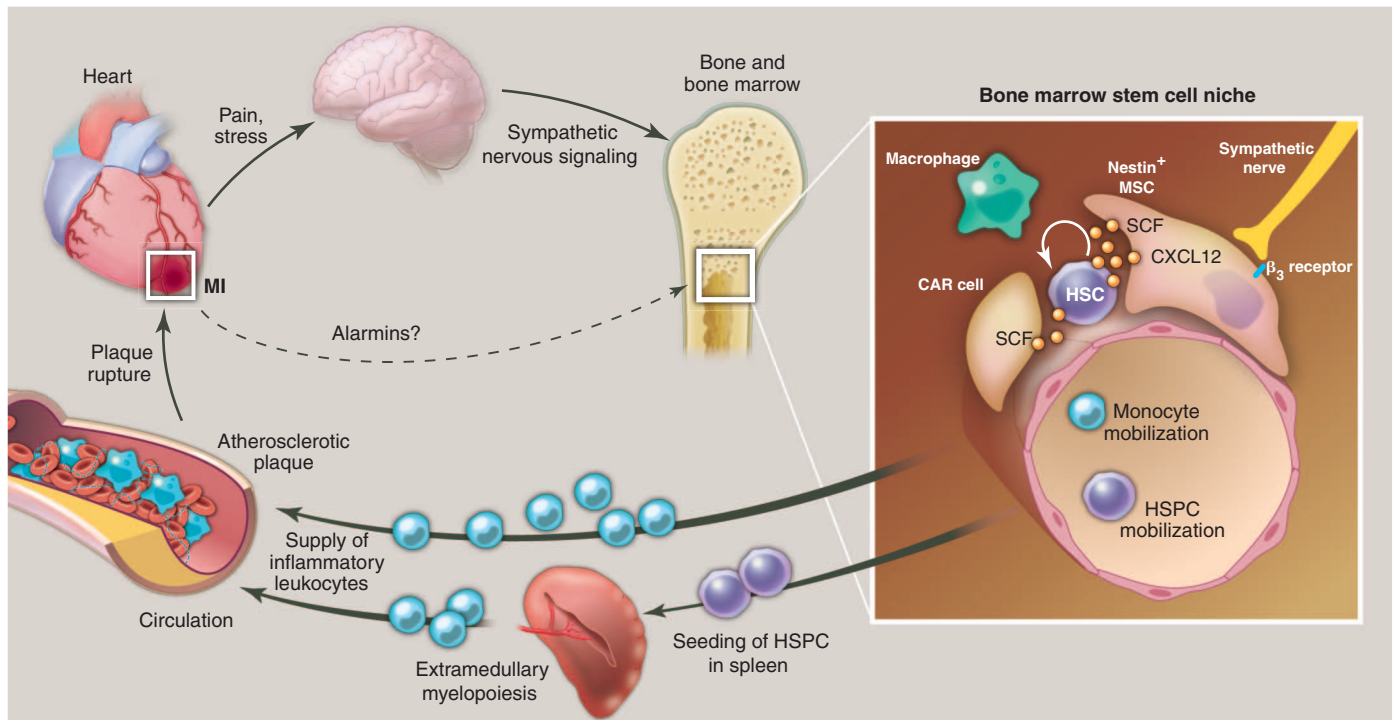


Fig. 3. Organ networks that lead to acceleration of atherosclerotic disease after MI. These events, observed in mice acutely after MI, lead to accelerated disease progression in atherosclerotic plaque. (**Inset**) Processes in the bone marrow microenvironment. Here, niche cells provide signals that regulate hematopoietic stem cell (HSC) activity and retention and leukocyte production. After MI, increased sympathetic nervous signaling releases noradrenalin in the bone marrow niche, which binds to β_3 adrenoceptors on niche cells. These withdraw the soluble factor CXCL12, which results in increased HSC activity

and emigration to extramedullary sites. Increased production of leukocytes then feeds an expanded pool of circulating monocytes that are recruited to atherosclerotic plaque in higher numbers, accelerating plaque growth and inflammation. This feedback loop may cause the high reinfarction rates observed clinically. The dotted arrow refers to currently unknown cross-talk between the cardiac wound and the hematopoietic system via alarmins (danger-associated molecular patterns, DAMPs), which may also activate the bone marrow after MI. CAR, CXCL12 abundant reticular cell.

disease research. Among the tools, animal models of atherosclerosis and MI, although imperfect, remain indispensable for identifying a biological process, testing its in vivo function and importance, and rationalizing its targeting in human disease.

Hallmarks of Leukocyte Function in Cardiovascular Disease

The leukocyte system's role in cardiovascular disease has several prevailing features. First, the system appears to be maladaptive. The danger signal during atherosclerosis or after MI is unlikely to be a microbe, yet leukocytes mobilize powerful antimicrobial and inflammatory molecules that cannot adequately handle either lipids or dying myocytes. Second, the system is heterogeneous. Macrophage abundance in atherosclerosis does not imply hierarchy, and, indeed, other leukocytes such as dendritic cells may be acting in parallel. Third, the system is collaborative: Leukocytes communicate through processes that elicit activation, differentiation, degranulation, and transmigration. Fourth, the system is competitive in that selective ablation of various same-class leukocyte subsets often reveals opposing effects on atherosclerosis. Fifth, the system is pervasive. Leukocytes accumulate in vascular lesions or the myocardium, but they are produced in other tissue and circulate in the blood. Sixth, the system is integrated. Leukocytes do not operate in isolation but belong to a network that connects organ systems (Fig. 3).

Concluding Remarks

Therapeutic approaches that target leukocytes to treat cardiovascular disease have not yet been realized, partly because the fundamental biology remains somewhat enigmatic (60). Important lingering questions include the following: What key triggers lead to leukocyte participation and activation? What are the initializing danger signals, modes of information transfer, and essential disease amplifiers? What defines the nature of the system's response (attack versus resolution)? What are the connecting points between organ systems, including lymphatic, endocrine, and nervous? Can we identify therapeutic targets that are efficacious yet specific enough to avoid collateral damage? Is it possible to target specific autoantigens such as LDL while preserving host defense? Answering these questions may be integral to steering leukocyte function away from self-destruction and toward physiological stability in order to durably prevent and suppress the devastating consequences of cardiovascular disease.

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REVIEW

Anti-Inflammatory Therapy in Chronic Disease: Challenges and Opportunities

Ira Tabas^{1*} and Christopher K. Glass^{2*}

A number of widespread and devastating chronic diseases, including atherosclerosis, type 2 diabetes, and Alzheimer's disease, have a pathophysiologically important inflammatory component. In these diseases, the precise identity of the inflammatory stimulus is often unknown and, if known, is difficult to remove. Thus, there is interest in therapeutically targeting the inflammatory response. Although there has been success with anti-inflammatory therapy in chronic diseases triggered by primary inflammation dysregulation or autoimmunity, there are considerable limitations. In particular, the inflammatory response is critical for survival. As a result, redundancy, compensatory pathways, and necessity narrow the risk:benefit ratio of anti-inflammatory drugs. However, new advances in understanding inflammatory signaling and its links to resolution pathways, together with new drug development, offer promise in this area of translational biomedical research.

All living organisms depend on the ability to protect themselves from exogenous pathogens (1) and to repair tissue damage that results from infection or trauma (Figs. 1, A and B, and 2A). Nonetheless, there are settings when the inflammatory response itself damages host tissue and causes organ dysfunction. In

one setting, there is an overly exuberant acute or subacute inflammatory response (cytokine storm) that occurs in response to pathogens (sepsis) or debris from damaged host cells (2). In another type of pathologic inflammation, a primary defect in the regulation of an inflammatory pathway triggers chronic disease. An example is

cryopyrin-associated periodic syndromes (CAPS), which are inflammasome diseases caused by mutations in NACHT-leucine-rich repeat protein 3 (3). A third class of chronic diseases is driven by pathologic inflammation but is not triggered by acute sepsis, acute tissue injury, or a primary defect in inflammation regulation. In these diseases, the inflammatory response does not eradicate the primary stimulus, as would normally occur in most cases of infection or injury, and thus a chronic form of inflammation ensues that ultimately contributes to tissue damage. In diseases

such as seropositive rheumatoid arthritis (RA), the inflammatory response is triggered by an exuberant, pathologic autoimmune cascade (4) (Fig. 1C). However, in other types of chronic inflammatory diseases, autoimmunity is not the key, primary pathogenic event (Fig. 2B) (5). Diseases in this category are often associated with aging and include atherosclerosis (Fig. 1D), obesity and insulin resistance, and certain neurodegenerative diseases such as Alzheimer's disease.

Improved understanding of the inflammatory response has led to important advances in the treatment of diseases with a primary defect in inflammation regulation, such as CAPS, and in autoimmune-induced inflammatory diseases, particularly seropositive RA and certain other rheumatoid diseases. Can these advances also be applied to other types of chronic diseases in which inflammation is an important driving force? In chronic autoimmune diseases, the mechanisms

linking the autoimmune trigger to the maladaptive inflammatory response are often better understood than in chronic inflammatory diseases with a nonautoimmune etiology. Moreover, autoimmune diseases are usually associated with life-changing, and often painful, symptoms on a day-to-day basis, which tends to increase patient acceptance of adverse effects of treatment. Nonetheless, even with this class of diseases, treatment is not ideal. The beneficial responses are variable, particularly when anti-inflammatory therapy is started after the disease has become established; long-lasting remissions are not common; and adverse effects, particularly in the area of compromised host defense, can be substantial (6). These problems are likely to be even more pronounced with complex and often indolent chronic disease processes in which the primary trigger is thought to be something other than autoimmunity.

The challenges in targeting inflammation in any chronic inflammatory disease lie in three properties that are characteristic of processes that are critical for evolutionary survival: redundancy, compensation, and necessity. Thus, inflammation is orchestrated by many molecules, and targeting one or a few may not be enough. Inflammation is also a finely tuned process that has inherent sensors and feedback pathways, and so inhibition of a critical component of inflammation may simply trigger a compensatory proinflammatory response involving another pathway. Finally, the inflammatory response is critical for host defense, and thus when the previous two challenges are successfully overcome, the risk:benefit profile is often unacceptable. With this background, we will first review the fundamental components of the inflammatory response, with emphasis on those components that contribute to redundancy and compensation and that are targets of currently available drugs or have promise for future therapeutic intervention. We will then highlight principles of anti-inflammatory therapy in chronic autoimmune inflammatory diseases, discuss challenges and ongoing attempts to target inflammation in other chronic inflammatory diseases, and raise prospects for future therapeutic directions.

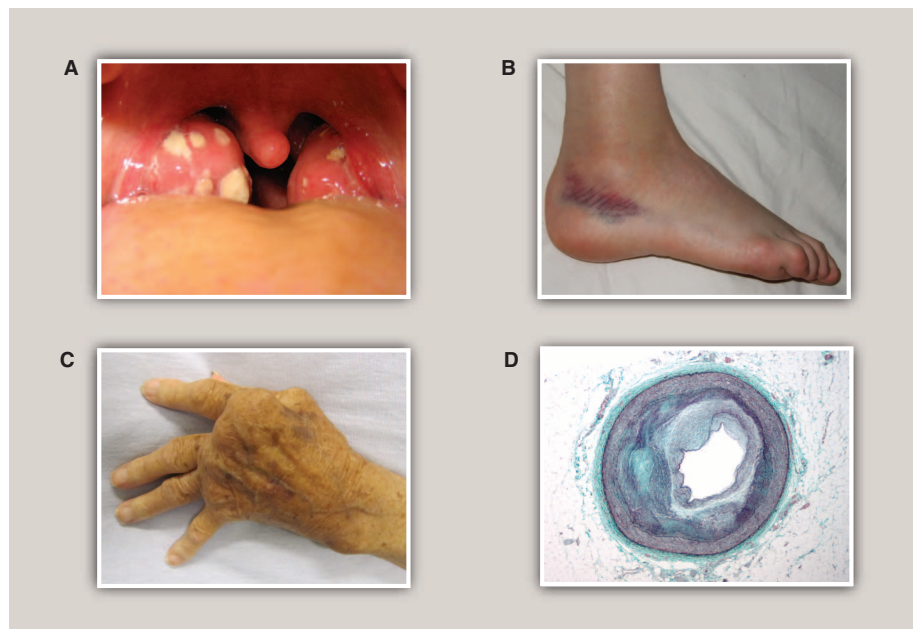


Fig. 1. Inflammation in infection, injury, and chronic disease. **(A)** Acute tonsillitis. White patches represent the accumulation of neutrophils in the tonsils in response to bacterial infection. The tonsils are swollen, erythematous, and painful. Normal tissue homeostasis is usually restored over the course of several days upon eradication of the infection. [Source: Michaelbladon/Wikimedia Commons] **(B)** Sprained ankle. An example of a sterile tissue injury. Swelling and redness result from the inflammatory response to tissue damage and hemorrhage. Normal tissue homeostasis is usually restored over a time course of weeks after clearance of dead or damaged cells and activation of tissue repair programs. [Source: Boldie/Wikimedia Commons] **(C)** Rheumatoid arthritis. An example of chronic inflammation in a disease triggered primarily by autoimmunity. Persistent inflammation, driven in part by $\text{TNF}\alpha$, results in severe tissue damage, joint destruction, and loss of function. [Source: James Heilman, MD/Wikimedia Commons] **(D)** Cross section of a coronary artery at the location of an atherosclerotic lesion. An example of a chronic inflammatory disease that is triggered initially by a process other than infection, tissue injury, or autoimmunity. Elevated levels of apolipoprotein B—containing lipoproteins in the artery wall induce a chronic inflammatory state characterized by activated endothelial cells and recruitment and activation of macrophages and other immune cells. As lesions evolve, inflammation fails to resolve in the setting of persistent arterial-wall lipoproteins. Nonresolving inflammation leads to cell death and necrotic core formation; cycles of extracellular matrix deposition and degradation; and calcification. [Source: Nephron/Wikimedia Commons]

Basic Principles of the Inflammatory Response and Inflammation Resolution

At the tissue level, acute inflammation is characterized by redness, heat, pain, and swelling, which result from local responses of immune, vascular, and parenchymal cells to infection or injury (Fig. 1, A and B). At a signaling level, infection or tissue damage is initially sensed by pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs) and/or damage-associated molecular patterns (DAMPs) (Fig. 3). Several classes of such receptors are now well characterized, exemplified by the Toll-like receptors (TLRs) and NOD-like receptors (NLRs) (7). Upon binding of PAMPs or DAMPs, PRRs engage signal transduction pathways that activate

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signal-dependent transcription factors such as nuclear factor κ B (NF- κ B) and activating protein 1 (AP-1). These factors act in a combinatorial and cell-specific manner to induce the expression of genes that initiate the inflammatory response (e.g., *TNFA*, *IL1B*, and *COX2*), exert antimicrobial functions (e.g., inducible nitric oxide synthase), and recruit additional immune cells (e.g., chemokines), thereby setting into motion both innate and adaptive immune responses. Notably, nearly all components of this early warning system are redundant. Furthermore, there are cytokine-mediated feed-forward loops that amplify the initial inflammatory response (Fig. 3). Epigenetic regulation is also important. For example, gain of histone H3 and H4 acetylation and loss of H3K27 and H4K20 methylation occur to facilitate activation of inflammatory response genes (8–11).

At a cellular level, acute inflammatory responses are characterized by marked temporal changes in quantities and characteristics of tissue immune cells. Under resting conditions, most tissues contain a population of resident macrophages that exhibit a deactivated or “resting” phenotype. There is substantial evidence that these cells play important roles in maintenance of normal tissue homeostasis. For example, lean adipose tissue contains deactivated macrophages, eosinophils, and regulatory T cells (T_{reg} s), all of which appear to contribute to normal insulin signaling (12–14). Upon infection with a bacterial pathogen, the initial phase of the acute inflammatory response is typically characterized by rapid neutrophil invasion (Fig. 2A). This early response subsequently gives way to a secondary phase characterized by recruitment of monocyte-derived macrophages, adaptive immune cells, and stromal cells. The combination of innate and adaptive immune responses serves to eradicate the infection but also results in collateral tissue damage, in part through the production of reactive oxygen species that are cytotoxic and secretion of proteases that degrade extracellular matrix.

At this stage, the inflammatory response transitions to an active resolution phase (Fig. 2A) (1, 15). Cellular processes involved in inflammation resolution include clearance of pathogens, pro-inflammatory debris, and cytokines; apoptosis of neutrophils and their non-inflammation-inducing removal by phagocytes (efferocytosis); egress of inflammatory macrophages and recruitment or phenotypic switching of macrophages to a pro-resolving phenotype; recruitment of T_{reg} s; activation of anti-inflammatory nuclear receptors; and overall repair and normalization of tissue architecture and function, including reestablishment of the vasculature. Many of these cellular processes are activated by and/or generate proresolving soluble factors produced by the inflammatory cells themselves or by cells recruited during the initial resolution phase. For example, prostaglandin E2 (PGE2), omega-3 fatty acids, and neutrophil-

derived microparticles in edema fluid can trigger resolution responses (16–18). Factors that mediate resolution include interleukin-10 (IL-10), transforming growth factor- β (TGF β), proresolving proteins such as annexin A1, and small lipid mediators called lipoxins, resolvins, protectins, and maresins that are derived from arachidonic

acid and omega-3 fatty acids through 5- or 15-lipoxygenase pathways (1, 15).

Principles of Anti-Inflammatory Therapy in Chronic Autoimmune Inflammatory Diseases

The understanding that the pathology of chronic autoimmune diseases is driven by maladaptive,

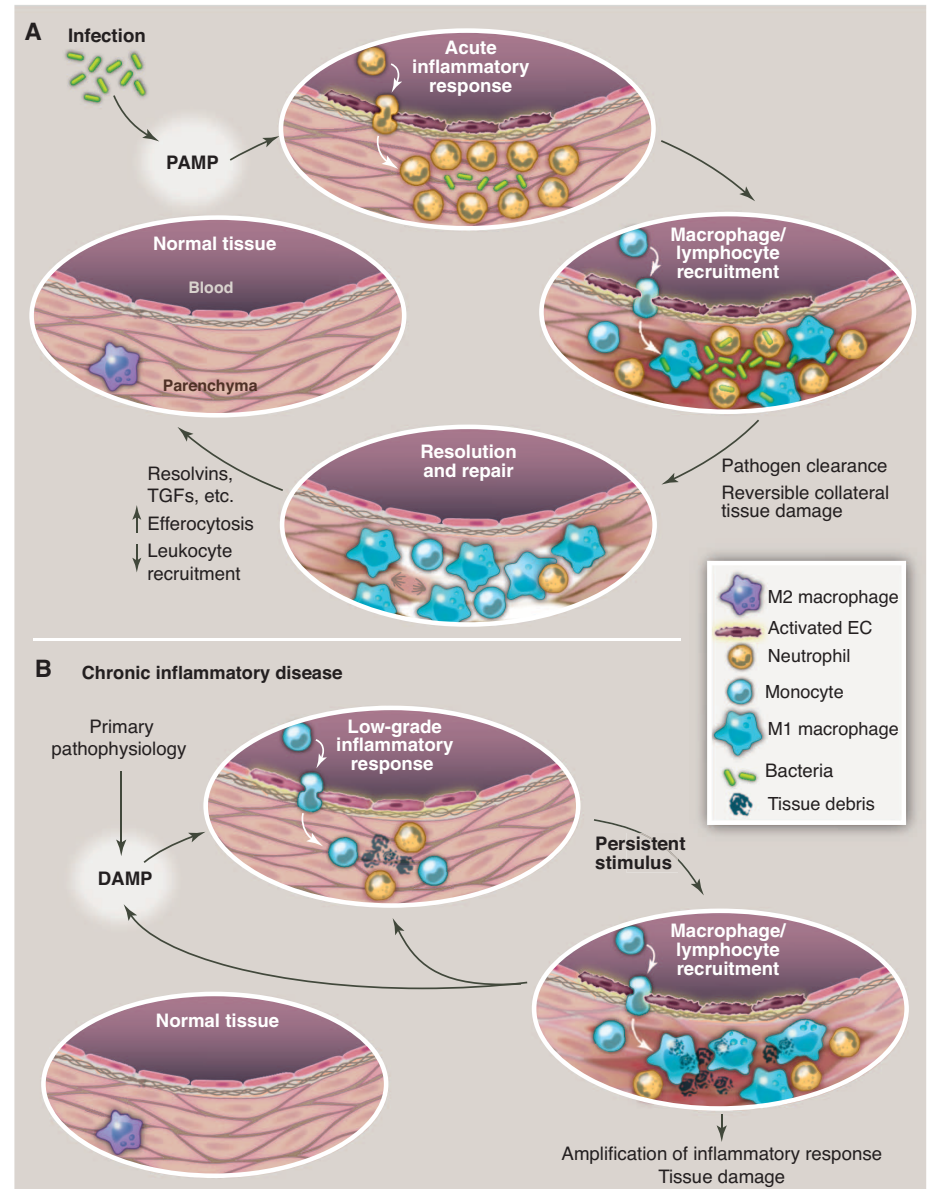


Fig. 2. Evolution of resolving versus nonresolving inflammation at a cellular level. **(A)** Typical features of a normal acute inflammatory response to infection that is detected by presentation of PAMPs to pattern recognition receptors. Eradication of the pathogen eliminates the stimulus, along with causing some reversible collateral tissue damage, and sets the stage for the resolution/repair phase, leading to restoration of normal tissue homeostasis. **(B)** Typical features of a chronic inflammatory disease caused by a nonimmune pathophysiological process that in one way or another triggers an initial sterile inflammatory response, often indolent and likely through production of damage-associated molecular patterns (DAMPs). This initial response then becomes amplified by cytokines and chemokines. Because this response does not eradicate the initial stimulus, persistent nonresolving inflammation occurs, ultimately resulting in tissue damage. The inflammatory response itself may positively influence the production of DAMPs, which provides an additional positive feedback loop. For example, in the case of atherosclerosis, reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) may modify subendothelial lipoproteins in a manner that amplifies their ability to promote inflammation.

nonresolving inflammation led to trials and then successful use of anti-inflammatory therapy, including nonsteroidal anti-inflammatory drugs (NSAIDs), glucocorticoids, and disease-modifying agents of rheumatoid diseases (DMARDs) (Fig. 3) (19). The first two classes are used mostly

to relieve symptoms, whereas DMARDs are distinguished by their ability to reduce or prevent tissue damage caused by the inflammatory attack, especially when used early in the course of the disease. Nonbiologic DMARDs, such as low-dose methotrexate (LD-MTX), suppress various inflam-

matory processes in multiple types of immune cells. Biologic DMARDs are genetically engineered recombinant proteins (e.g., humanized antibodies) that target specific inflammatory molecules or their receptors or signaling pathways—for example, tumor necrosis factor- α (TNF α), IL-1 β , and the IL-1 receptor (Fig. 3). Combination therapy with a nonbiological plus a biological DMARD is commonly used and very effective, but, apropos of the “necessity” challenge, combinations of different classes of biologic DMARDs are not used due to high risk of infection.

An example of how the development of a biologic DMARD met some of the challenges of anti-inflammatory therapy can be appreciated by briefly reviewing the history of antibody to TNF α (anti-TNF α) as therapy for antibody to citrullinated protein (ACPA)-positive RA (6). The challenge of redundancy and compensation, with many inflammatory cytokines and other mediators present in the arthritic joints, was daunting and an early deterrent to drug development. Cleverly conducted ex vivo rheumatoid synovium experiments revealed, however, that many of the inflammatory mediators were in the same pathway and that TNF α was a proximal trigger. Thus, anti-TNF α blocked inflammatory cell production of many other cytokines in addition to TNF α and, importantly, chemokines. The decrease in chemokines then amplified the beneficial effect by reducing trafficking of the inflammatory cells to the joints. Subsequent studies in mouse models confirmed this hierarchy. In striking contrast, therapeutic targeting of another inflammatory mediator in ACPA-positive RA, the mitogen-activated protein kinase p38, was found to be too transient to be useful, suggesting that the challenges of redundancy and/or compensation were not overcome in the targeting of this particular pathway (20). Moreover, anti-TNF α is not effective in certain other inflammatory conditions, such as septic shock, and even appears to be harmful in multiple sclerosis, perhaps because inhibiting this cytokine compromises the transition to resolution in these settings (21, 22).

Even in the case of successful anti-TNF α therapy, the response is variable, so we can learn lessons from its limitations (6). First, patient benefit of anti-TNF α is better in early disease than in late disease, where irreversible tissue damage has already occurred. This property of anti-inflammatory therapy is inherent to all chronic inflammatory diseases. Therefore, methods to identify early disease, such as biomarkers and imaging techniques, and finding drugs that can be tolerated for very long periods of time are important in the overall design of anti-inflammatory strategies for chronic diseases. Second, despite the fact that anti-TNF α has been relatively successful in the face of redundancy and compensatory pathways, these challenges still exist. Finally, basic principles of necessity dictate that the more potent the anti-inflammatory therapy, the greater chance for adverse effects related to host defense.

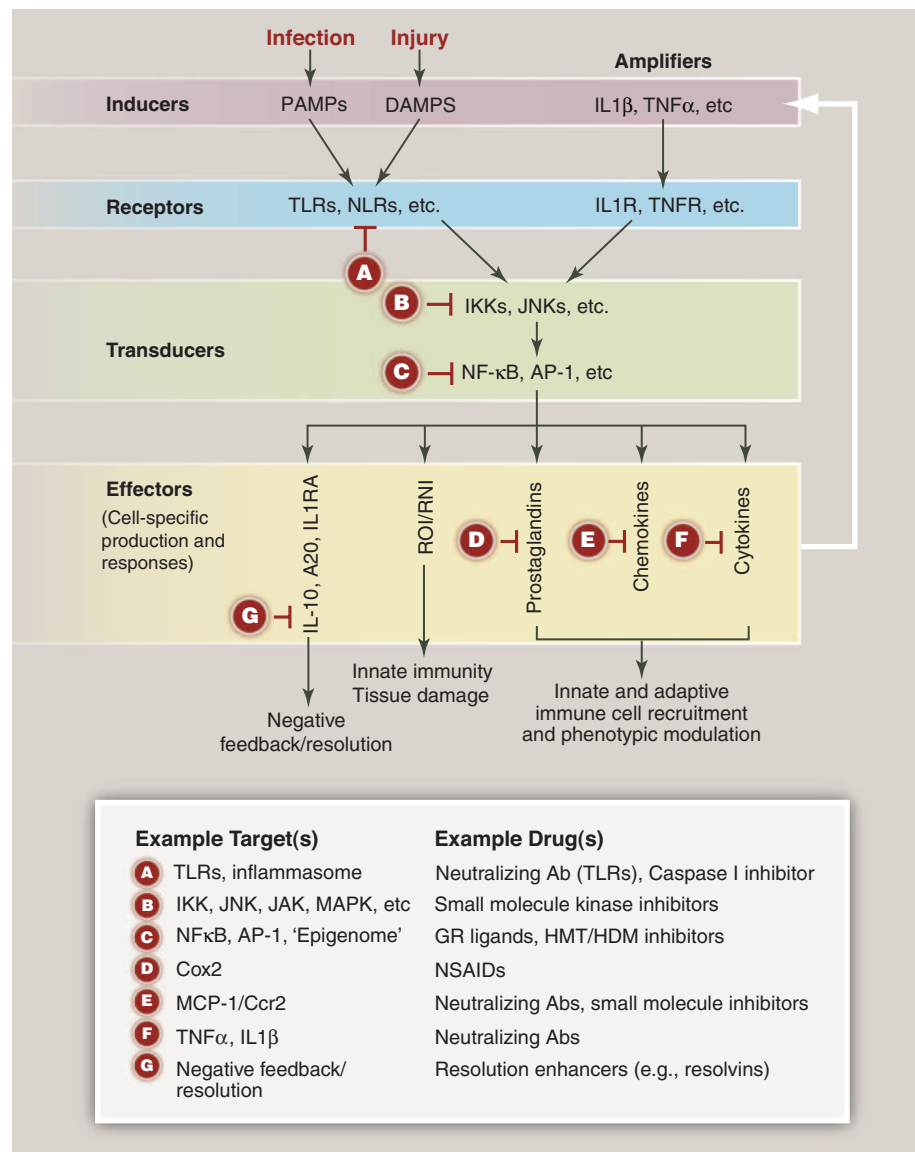


Fig. 3. Inflammation at a signaling level and candidate therapeutic targets. Inflammation is typically initiated by pattern recognition receptors, such as TLRs and NLRs, that recognize PAMPs and/or DAMPs. These receptors typically couple to signal transduction pathways that activate latent transcription factors that include members of the NF- κ B and AP-1 families. These factors in turn act in a combinatorial and cell-specific manner to induce the expression of a large number of genes that exert antimicrobial activities—e.g., generate ROI and RNI. Chemokines regulate the recruitment of additional immune cells. Production of bioactive lipids, such as prostaglandins, also regulates pro- and anti-inflammatory cell functions. Expression of inflammatory cytokines provides a feed-forward loop for amplification of the initial response. The production of anti-inflammatory/resolution mediators, such as IL-10 and PGE₂, in response to proinflammatory signals suggests that resolution programs are an inherent aspect of inflammation. Letters in red “stop signs” represent examples of points in proinflammatory signaling pathways that are existing or potential targets for therapeutic intervention. Ab, antibody; GR, glucocorticoid receptor; HDM, histone demethylase; HMT, histone methyltransferase; IKK, I κ B kinase; MAPK, mitogen-activated protein kinase; MCP1, monocyte chemoattractant protein-1; TNFR, tumor necrosis factor receptor; Cox2, cyclooxygenase 2; NSAIDs, nonsteroidal anti-inflammatory drugs.

Thus, it is not surprising that patients taking anti-TNF α therapy are at increased risk for infections. In the end, the devastating nature of RA creates a risk:benefit ratio that more often than not favors drug treatment.

Targeting Inflammation in Chronic Diseases with an Inflammatory Component Not Triggered by Autoimmunity

Chronic diseases associated with an inflammatory component not directly induced by an autoimmune process are the most common diseases of aging and represent our greatest health threats (3). These include most forms of cardiovascular disease, type 2 diabetes, and virtually all neurodegenerative diseases. In each case, a nonautoimmune primary pathological process—for example, excess subendothelial apolipoprotein B-containing lipoproteins, saturated fatty acids, or formation of protein aggregates, respectively—results in the generation of DAMPs that are detected by PRRs (Fig. 2B). Moreover, the inflammatory response itself may amplify the production of disease-specific DAMPs, resulting in positive-feedback loops that accelerate the underlying disease process. For example, inflammation promotes formation of oxidized phospholipids that may serve as important DAMPs in atherosclerosis (23) and may enhance the formation of β -amyloid and tau aggregates in Alzheimer's disease (24). As with autoimmune diseases, inhibition of inflammation could reduce the rate of disease progression to the point of substantial clinical benefit despite not altering the underlying pathogenic process. In contrast with primary inflammatory or autoimmune diseases, however, there is little evidence as yet for efficacy of this approach in humans.

Each of the diseases in this category has unique therapeutic opportunities and challenges. For example, the evaluation of anti-inflammatory drugs for type 2 diabetes, as compared with atherosclerosis and, especially, Alzheimer's disease, is more feasible in terms of end-point analysis (fasting blood sugar, hemoglobin A1c, and plasma insulin levels) and may not be as demanding in terms of the necessity for early-stage treatment (25). For a discussion of targeting inflammation in metabolic and neurodegenerative diseases, see the Reviews in this issue by Odegaard and Chawla (26) and Aguzzi *et al.* (27), respectively. The focus here will be on atherosclerosis, which is an important and instructive example of the challenges in this area (28).

Subendothelial retention of apolipoprotein B-containing lipoproteins triggers a maladaptive, nonresolving inflammatory response that drives atherogenesis [see the Review in this issue by Swirski and Nahrendorf (29)]. Here, we emphasize a few points that are relevant to the discussion that follows. First, the statin class of drugs are used in almost all subjects at high risk for atherosclerotic disease. Thus, anti-inflammatory therapy would be used mostly in combination with statins and would have to show effectiveness great-

er than that with statins alone, which themselves may have some anti-inflammatory effects (30). Second, although early use of anti-inflammatory therapy might be most effective in combating atherosclerosis before certain aspects of the pathology become irreversible, this would require a very long treatment period and may therefore have an unacceptable risk:benefit ratio. Conversely, although the risk:benefit ratio might be most acceptable for shorter-term treatment of advanced atherosclerosis, it is precisely in this situation that anti-inflammatory therapy would be least effective. In this setting, the value may lie in preventing the progression of earlier lesions that are known to coexist with advanced lesions. Third, it is important to consider the possibility of unique adverse effects of targeting inflammation in atherosclerosis. For example, recurrence of a myocardial infarction (MI, or heart attack) is particularly prominent in the year following an initial MI. This heightened risk may be driven in part by a post-MI monocytosis, but inhibiting monocytosis through anti-inflammatory therapy may delay tissue repair of the infarcted myocardium itself (31). Furthermore, inhibition of a master regulator of inflammation, NF- κ B, or deletion in macrophages of tumor necrosis factor receptor-associated factor 6 (TRAF6), which is an adaptor of IL-1R and TLRs, actually increases atherosclerosis in mouse models by mechanisms that appear to involve compensatory signaling (32, 33). On the other hand, a possible additional benefit of targeting systemic inflammation in atherosclerosis may be its effects on obesity and insulin resistance (34), which itself may be lessened by anti-inflammatory therapy (26).

With these considerations in mind, how might the inflammatory nature of atherosclerosis lead to new therapeutic advances? First and foremost, atherosclerosis has a property that is lacking in most chronic inflammatory diseases, namely, the potential for removing the inflammatory stimulus. As with gout, where lowering of uric acid levels can help prevent the formation of uric acid crystals in joints and thereby reduce inflammation, lowering of plasma low-density lipoprotein (LDL) can prevent subendothelial retention of lipoproteins and thereby decrease inflammatory atherosclerotic disease (35). However, the goal of therapeutically decreasing plasma LDL to low enough levels and early enough in the disease to eliminate atherosclerosis has not yet been achieved, so other treatment options are being sought.

In this context, investigators are beginning to ask whether drugs used for chronic primary inflammatory diseases might be useful to prevent atherosclerotic disease, and they have recently turned their attention to DMARDs. The genetic and pharmacologic targeting of many individual inflammatory processes and molecules in mouse models of atherosclerosis leads to decreases in aortic atherosclerosis. However, it has been argued that although testing new antiatheroscle-

rosis drugs in vitro and in preclinical models in vivo is a necessary first step, it is often a poor way to predict success in vascular disease in humans. Indeed, clinical trials may be the best way to investigate proof-of-concept usefulness (28). In that spirit, two human DMARD coronary artery disease (CAD) trials are now being initiated. One will investigate the use of LD-MTX, which was shown in post hoc analyses of several RA and psoriatic arthritis trials to have potentially beneficial effects on cardiovascular disease (36). The other trial will test a humanized monoclonal antibody against IL-1 β (canakinumab), which is being used or evaluated for two types of inflammasome/IL-1 β -mediated diseases, CAPS and gout, respectively (37). Part of the rationale for IL-1-targeted therapy is evidence for inflammasome activation in murine atherosclerosis, perhaps triggered by cholesterol crystals (38), and the success of targeting IL-1 β in these mouse models.

It is instructive to view these two proposed trials in the context of the successful development of anti-TNF α and LD-MTX for ACPA-positive RA and the challenges of redundancy, compensation, and necessity. Atherosclerotic lesions, like the RA synovium, express many cytokines and chemokines that have been implicated in the pathogenesis of atherosclerosis. As noted above, TNF α has a distinct role in synovial inflammation in that it controls the production of many other cytokines and, more generally, leukocyte trafficking. Whether IL-1 β has a similar upstream role in human atherosclerosis is unknown, and the successful use of canakinumab in CAPS and gout may be because these diseases are primary inflammasome/IL-1 β diseases. If the role of IL-1 β is not particularly dominant in atherosclerotic plaque progression in humans, the benefit above that seen with statins alone may be difficult to detect. Moreover, although neutralization of IL-1 β diminishes atherosclerotic lesion size in mice, deletion of IL-1 α/β receptors was found to actually worsen plaque stability in a mouse model of advanced atherosclerosis through effects on the extracellular matrix (39). Finally, the potential benefit of long-term use of canakinumab in humans at high risk for atherosclerotic vascular disease must be substantial enough to counter the increased risk of infection, which was 67% in a recently conducted 2-year CAPS trial vs. 25% for placebo.

Despite the aforementioned favorable signal from post hoc analyses of previous clinical trials, there is more uncertainty with LD-MTX because the exact mechanisms of action are not completely understood. To the extent that the benefit of LD-MTX may rely, at least in part, on adenosine-mediated anti-inflammatory signaling, mouse atherosclerosis studies would predict promise (40, 41). Perhaps a greater challenge will be related to adverse effects. Although LD-MTX is considered to be a relatively safe and well-tolerated drug, it has several mild and usually self-limited

nuisance-type side effects, including nausea, stomatitis, and fatigue, and long-term use has rarely been associated with more important adverse effects, such as hepatotoxicity, pulmonary disease, and infection (42). If LD-MTX is effective in atherosclerosis but requires long-term use, these side effects might become more important. Moreover, the risk of liver injury and infection is increased in subjects with type 2 diabetes (42, 43), which represents a sizable percentage of the high-risk CAD population that would be considered for this drug. In the end, it will be important to consider these principles both for the monitoring and evaluation of the canakinumab and LD-MTX trials and in the design of and decision to move forward with new trials in this arena.

Future Directions

Ongoing efforts to widen the benefit-to-risk window of anti-inflammatory therapy in chronic diseases will require efforts on a number of complementary fronts. To the extent that there is the potential to remove the inflammatory stimulus in these diseases, as there is in atherosclerosis (atherogenic lipoproteins) and obesity (nutrient excess), ongoing efforts in this area are crucial. For example, in atherosclerosis, there may be innovative therapeutic approaches to prevent the retention of atherogenic lipoproteins in addition to lowering plasma LDL (44). In general, however, these goals have been difficult to attain even where they are theoretically possible, and it is not yet feasible in other chronic diseases, such as neurodegenerative disease.

With regard to DMARDs, a number of future developments may increase their promise in a wider array of chronic inflammatory diseases. Data from human genetic studies that link specific inflammatory genes to CAD may be helpful in identifying useful drug targets (45). In addition, detailed, disease-specific mechanistic studies will help identify targets that are upstream in signaling cascades, which will help guard against redundancy, and whose inhibition is less likely to trigger compensatory responses, compromise host defense, and suppress the transition to resolution. Some of these goals may be achievable through the use of new formulations that target DMARDs to the site of inflammation, thus minimizing systemic adverse effects.

Translating these advances into therapy will benefit from simultaneous progress in developing new classes of drugs that target inflammation. Recent examples include an inhibitor of inflammatory Janus kinase (JAK) signaling (tofacitinib), which has recently been shown to be effective in RA and ulcerative colitis (46); a potent and site-selective adenosine A(2A) receptor activator that requires ectonuclease-mediated activation at the site of inflammation (47); and drugs that target inflammatory fibroblasts, which have shown promise in preclinical models of RA and may be useful in other inflammatory diseases depending

on the roles of these cells (48). There is also substantial evidence supporting the feasibility of using antisense oligonucleotides (ASOs) to target specific RNAs in humans (49). The primary attraction of ASO technology is that, in principle, it enables knockdown of any RNA-encoded target, ranging from proteins that are very difficult to target with drugs to noncoding RNAs. In addition, the sequence precision of the ASO targeting mechanism enables highly related members of druggable families to be specifically targeted in a manner that could be difficult to achieve with small molecules. For example, an ASO specific for MKK7 reduced phosphorylation of c-Jun N-terminal kinase (JNK) in synoviocytes and reduced inflammation and disease severity in a mouse model of arthritis (50). Another strategy would be to use recent advances in histone modifications. Examples include inhibitors of the bromodomain and extraterminal domain family of proteins (I-BET), which block the binding of transcription initiation mediators called BET proteins to acetylated histones and thereby inhibit transcription. The inhibitors exert remarkably selective effects on gene expression *in vitro* and *in vivo*, acting to suppress a subset of inflammatory response genes in activated macrophages and conferring protection against endotoxin-induced shock (51). Another example of epigenetic-based therapy is a druglike compound that suppresses lipopolysaccharide-induced cytokine production in macrophages by inhibiting a specific subfamily of histone demethylases (52).

An alternative strategy to inhibiting inflammation would be to commandeer nature's own anti-inflammatory mechanisms to induce a "dominant" program of resolution. Examples include activators of liver-x receptors (LXRs), which regulate inflammation and lipid metabolism and have shown benefit in mouse models of atherosclerosis and Alzheimer's disease (53–56), and activators of peroxisome proliferator-activated receptor (PPAR) γ , which may be able to reprogram white adipose tissue macrophages and promote T_{reg} development to improve insulin resistance in obese adipose tissue (12). Recent studies suggest new strategies to enhance therapeutic actions of these receptors and reduce side effects (57, 58). Future therapies may be able to take advantage of two key suppressors of inflammation, IL-10 and T_{regs} (59). Using atherosclerosis as an example, IL-10 and T_{regs} have marked antiatherosclerotic effects in mouse models (60, 61). Long-duration systemic IL-10 therapy would likely have substantial adverse effects, but nanoparticle-targeted delivery of IL-10 to atherosclerosis lesions may be both feasible and effective (62). Antiatherogenic T_{regs} might be inducible through the use of athero-specific dendritic cell vaccine strategies (63), perhaps in combination with agents that activate endogenous cell biological processes involved in the stability and function of T_{regs} and/or in the feedback suppression of inflammatory T effector cells (64, 65).

Another line of attack may be therapeutic administration of lipid mediators of inflammation resolution, particularly in view of evidence that chronic inflammatory diseases may lower the levels or actions of these molecules (66). In this regard, resolvin E1 (RvE1) has shown benefit in preclinical models or clinical trials of a number of chronic inflammatory diseases, including asthma, rheumatoid arthritis, atherosclerosis, and obesity-related adipose inflammation (www.resolvyx.com) (67), and neuroprotectin D1 protected human neurons from beta-amyloid-induced inflammation and cell death *in vitro* (68). A key principle in the therapeutic use of inflammation resolution mediators is that they are predicted not to compromise host defense. In fact, recent work in mice has shown that RvD2 actually promotes host defense during sepsis (69).

Finally, it is important to consider how one could noninvasively monitor the anti-inflammatory or proresolving actions of drugs that target the inflammatory component of chronic diseases. This is particularly important in diseases like atherosclerosis where the actual clinical end points themselves are delayed, sporadic, and often devastating. To the extent that the drugs affect systemic inflammation, measurement of plasma cytokines and acute-phase reactants, including TNF α , IL-6, C-reactive protein, and fibrinogen, can be monitored. Monitoring inflammation at the site of disease would be particularly useful, and thus there may be a niche in this arena for techniques such as 18F-fluorodeoxyglucose positron emission tomography and fluorine-19 magnetic resonance imaging (47, 70). However, all these methods lack specificity and may also lack sensitivity when it comes to specific therapeutic areas, such as those involved in enhancing inflammation resolution. Thus, efforts to target the inflammatory and nonresolving components of chronic diseases, particularly those with delayed signs or symptoms, must be accompanied by the development of noninvasive methods to monitor the effectiveness of these new strategies.

In conclusion, the past two decades have provided a wealth of information on how maladaptive, nonresolving inflammation drives a number of widespread chronic diseases in which infection, primary defects in inflammation regulation, or autoimmunity are not the primary pathophysiologic process. Although this knowledge has the potential to open up vast opportunities for new therapeutic advances, the nature of the inflammatory response as a complex system that is critical for normal physiology renders this promise challenging. In particular, the challenges of redundancy, compensation, and necessity often create a very narrow risk:benefit window. Nonetheless, new knowledge about inflammatory signaling, particularly in the areas of endogenous homeostatic pathways and inflammation resolution, provide the promise for new therapeutic options that can successfully meet these challenges.

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REVIEW

Pleiotropic Actions of Insulin Resistance and Inflammation in Metabolic Homeostasis

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Metabolism and immunity are inextricably linked both to each other and to organism-wide function, allowing mammals to adapt to changes in their internal and external environments. In the modern context of obesogenic diets and lifestyles, however, these adaptive responses can have deleterious consequences. In this Review, we discuss the pleiotropic actions of inflammation and insulin resistance in metabolic homeostasis and disease. An appreciation of the adaptive context in which these responses arose is useful for understanding their pathogenic actions in disease.

Humans have evolutionarily confronted three primary killers: starvation, infection, and predation. Through modern agricul-

ture, hygiene, and our relatively recent elevation to top predator status, we have made remarkable progress in mitigating these, only to find new, evolutionarily novel threats taking their place—principally, cardiovascular disease, diabetes, and cancer. Unmasked by our successes against more ancient challenges, these modern diseases represent a rapidly increasing share of human morbidity and mortality in both relative and absolute terms and threaten the gains in life expectancy already achieved. In recent years, obesity has emerged as the driving force behind these disturbing trends.

From 1980 to 2008 alone, the number of overweight individuals worldwide doubled to more than half a billion people, eclipsing the number of underweight individuals for the first time in history and driving the obesity-attributable death rate to ~3 million per year (1). More poignantly, even a spare handful of extra pounds in midlife is associated with a 20 to 40% increase in all-cause mortality, obesity with an ~100% increase, and morbid obesity with an ~300% increase (2). Despite such chilling numbers, the true effect of obesity is still likely to be understated.

Notwithstanding its catastrophic consequences, obesity's importance went long unappreciated because it acts less by overt effect than by promoting/exacerbating cardiovascular disease, diabetes, and cancer, among other diseases. Indeed, the mechanistic links between obesity and better-established pathologies have been hotly investigated over the past two decades. This Review primarily explores the cellular and molecular connections between chronic low-grade inflammation, insulin resistance, and obesity-induced metabolic disease. We begin by summarizing our current mechanistic understanding of obesity-induced insulin resistance and then discuss the importance of the histologic and evolutionary context within which it arises. Specifically, we present the argument that key mediators of obesity-induced metabolic disease, such as insulin resistance and inflammation, are evolution-

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arily conserved adaptive traits with maladaptive effects in the modern obesogenic environment.

Obesity-Induced Insulin Resistance

The fundamental characteristic of obesity is chronic imbalance between caloric intake and energy expenditure, resulting in the storage of excess nutrients in white adipose tissue (WAT) (3). In lean individuals, professional metabolic tissues such as WAT, liver, and skeletal muscle readily buffer excess nutrients by storing them as triglycerides and glycogen. With chronic over-nutrition, however, the storage capacity of professional metabolic tissues is eventually exceeded. This causes intracellular buffering mechanisms within dedicated nutrient-storing cells to break down and excess nutrients to overflow into physiologic compartments that are ill equipped for substrate handling. Consequently, both professional metabolic and bystander tissues are exposed to super-physiologic levels of metabolic substrates, resulting in cell-intrinsic and -extrinsic dysfunction (4, 5). The primary cell-intrinsic dysfunctions include lipid dysregulation (for example, accumulation of intracellular diacylglycerols, saturated fatty acids, and ceramides), abnormal intracellular protein modification, mitochondrial dysfunction/oxidative stress, and endoplasmic reticulum/membrane stress. Ectopic lipid deposition, abnormal extracellular protein modification (such as hemoglobin A1c and advanced glycation end-products), and adipokine dysregulation represent the major cell-extrinsic pathways (Fig. 1). With persistent imbalance between energy intake and expenditure, these processes escalate and eventually lead to adipocyte death, as is observed in obese WAT (6).

Obesity-induced cellular dysfunction activates a diverse range of stress-responsive and counter-regulatory signaling pathways, including activation of Jun N-terminal kinases (JNK), inhibitor of nuclear factor κ B (I κ B) kinase β (IKK β), endoplasmic reticulum-to-nucleus signaling 1 (IRE-1), mammalian target of rapamycin (mTOR), extracellular signal-regulated kinases (ERKs), protein kinase C θ (PKC θ), suppressor of cytokine signaling (SOCS) proteins, and RNA-activated protein kinase (PKR) (4, 5, 7–11). Although a detailed discussion is beyond the scope of this Review, these pathways collaborate to produce two metabolically important effects. First, each pathway converges on and inhibits insulin signaling

ways, primarily through serine phosphorylation of IRS (insulin receptor substrate) proteins, which blunts insulin action in stressed target tissues and stems the influx of nutrients into already overwhelmed cells (Fig. 1). Second, these signals converge on two main inflammatory signaling pathways, JNK and IKK β , to initiate, support, and augment an inflammatory response within metabolic tissues (Figs. 1 and 2). In parallel with these actions, dysregulated nutrient intake can also bypass cellular stress responses entirely and trigger inflammatory activation through a variety of mechanisms, including triggering of innate immune receptors (for example, saturated fatty acids–fetuin A ligation of Toll-like receptor 4) (12), increased gut-derived lipopolysaccharide (LPS) translocation, and intestinal dysbiosis (13).

Despite using similar pathways and mediators, the inflammatory response in obesity differs substantially in duration and intensity from that observed in the more familiar setting of infection. For instance, infectious inflammation involves

parameters, such as body weight and leukocyte activation, in obesity (14).

Inflammation: A Link

Inflammatory activation within metabolic tissues potentiates insulin resistance and metabolic disease by three principal means (Fig. 2) (15). First, inflammatory signaling pathways, such as cellular stress-induced and counter-regulatory cascades, inhibit insulin signaling through direct inhibitory serine phosphorylation of IRS proteins by JNK and IKK β (9). Second, secreted inflammatory mediators [such as the chemokine (C-C motif) ligand 2 (Ccl2), Ccl5, and Ccl8, which are produced by lipid-engorged adipocytes] recruit circulating leukocytes (such as Ly6C^{Hi} monocytes) to stressed tissue to augment the inflammatory signaling and tissue remodeling capacity of tissue-resident cells (15). Third, secreted inflammatory mediators communicate insulin resistance systemically as well as locally to recruited leukocytes, biasing them toward an inflammatory phenotype (11, 15). Although the first effect involves professional nutrient handling cells, such as adipocytes, hepatocytes, and skeletal myocytes, the latter two establish a stable, feed-forward signaling loop in which tissue-resident and recruited leukocytes sustain and augment both local and systemic inflammation and insulin resistance.

As might be expected, activation of this inflammatory circuit is accompanied by shifts in leukocyte populations and activation status that have profound effects on systemic metabolic parameters. In obesity, for example, macrophages increase from ~10% of all adipose tissue cells to over 50%, shift from an even to a clustered topographic distribution (primarily because of the appearance of necrotic adipocytes), and swap an immunoregulatory M2 phenotype (CD206⁺, Arg1⁺, and CD301⁺) for a proinflammatory, M1 bias [CD11c⁺, nitric oxide synthase 2⁺ (NOS2⁺), and tumor necrosis factor- α ⁺ (TNF- α ⁺)] (16–19) (Box 1). Adipose tissue-associated lymphocytes undergo a similar

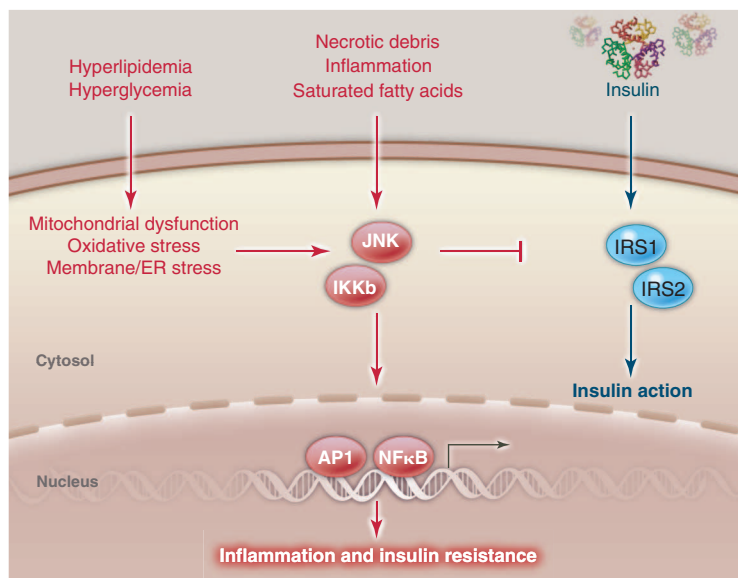


Fig. 1. Inflammatory signaling pathways link nutrient excess to insulin resistance. Insulin's presence at the cell surface is transduced to cytoplasmic and nuclear responses by tyrosine phosphorylation of IRS-1 and IRS-2. Serine phosphorylation of these same proteins by JNK and IKK β , however, potentially inhibits insulin signaling. Many diverse cell-intrinsic and -extrinsic sequelae of chronic nutrient excess activate these signaling pathways, directly linking overfeeding to insulin resistance. Furthermore, JNK and IKK β activation triggers inflammatory cytokine production, further activating JNK/IKK β in an autocrine and paracrine manner and reinforcing insulin resistance. ER, endoplasmic reticulum; AP 1, activator protein 1.

short-lived, high-amplitude responses, whereas metabolic inflammation, like other chronic inflammatory conditions, smolders at low levels for years to decades. Although the underlying mechanisms contributing to these differences are not entirely clear, it seems likely that hormonal or epigenetic programming may permanently reassign certain systemic and tissue-specific pa-

reorganization with the small T helper 2 (T_H2)/regulatory T cell (T_{reg} cell)-dominated repertoire associated with lean individuals giving way to a much larger and more inflammatory T_H1/CD8-dominated population in the obese (20–22). Furthermore, interleukin-4 (IL-4)-expressing eosinophils resident in lean WAT are displaced by waves of ingressing neutrophils,

mast cells, and B cells in obese individuals (23–25).

The marked shift in leukocyte population represents a key mechanistic link in the progression from overfeeding-related cellular stress to metabolic dysregulation to frank disease (26). In lean mice, alternative M2 macrophages (Box 1), T_{reg} cells, eosinophils, and invariant natural killer T cells collaborate to maintain an insulin-sensitive, tolerogenic immune environment (Fig. 3) (17, 20, 23, 27–31). Functional depletion of any of these leukocyte lineages disrupts this collaborative effort, destabilizing the anti-inflammatory environment, negatively affecting adipocyte insulin signaling, and exacerbating the deleterious effects of high-fat diets. Supplementation of any one of these cellular constituents has the opposite effect. Through the mechanisms discussed above, obesity reorganizes the leukocyte landscape into an insulin resistant, pro-inflammatory milieu in which the tolerogenic leukocyte network is disrupted and replaced with inflammatory M1 macrophages (Box 1), $CD8^+$ T cells, and T_H1 cells (Fig. 3). Functional depletion of any one of these lineages weakens this inflammatory influence, lessening the effects of obesogenic diets, whereas their supplementation exacerbates inflammation and disease (21, 22, 32). Furthermore, even interventions that prevent or augment recruitment of new leukocytes to WAT without influencing the existing leukocyte populations (for example, abrogation or amplification of the $Ccl2$ - $Ccr2$ chemotactic axis) can dramatically modulate obesity-associated insulin resistance (33, 34).

Although WAT, the most structurally dynamic nutrient-storing tissue, demonstrates dramatic alterations in obesity, similar leukocyte shifts take place in other metabolically important organs as well. For example, obesity precedes restructuring of pancreas- and liver-associated leukocyte populations (although the latter occurs without substantial change in macrophage number) (28, 35, 36), whereas brain and skeletal muscle acquire inflammatory microenvironments without substantial numeric alterations in leukocyte complements (37, 38). Coincident with this shift, the major tissue targets of insulin action begin to advertise the hallmarks of insulin resistance: increased triglyceride lipolysis in WAT; increased insulin production in pancreatic islets; elevated gluconeogenesis, glycogenolysis, and lipogenesis in the liver; decreased insulin-stimulated glucose disposal in skeletal muscle; and decreased satiety signaling in the brain. Shifts in the leukocyte populations occurring in these organs have systemic importance similar in scope to those occurring in adipose tissue. For example, liver-selective abrogation of alternative M2 macrophage activation results in increased obesity and systemic metabolic disease in response to high-fat diet (27, 28), a phenotype similar to that seen in whole-animal abrogation of the alternative M2 program (17, 29, 30, 39). These data support an

integral role for resident leukocytes in local, tissue-specific manifestations of metabolic disease and indicate that leukocyte dysregulation in even one tissue bed increases systemic susceptibility to metabolic disease.

Although obesity-induced metabolic disease promotes and exacerbates pathology through numerous disease-specific mechanisms, most pathology ultimately arises from obesity's characteristic milieu of chronic low-grade inflammation and insulin resistance. For example, obesity has been recognized for decades as an important risk factor for cardiovascular disease; however, only recently has that risk been mechanistically understood as a result of obesity's accompanying inflammation and insulin resistance (40). Similarly, hyperlipidemia—another well-described contributor to cardiovascular disease—arises as a consequence of inflammatory insulin resistance through increased adipose tissue lipolysis and hepatic lipogenesis. Even the biomechanical dynamics of cardiovascular disease—atherosclerotic plaque formation, remodeling, and rupture—are influenced by the inflammatory milieu (40). Indeed, the efficacy of some anti-hyperlipidemic therapies correlates with their immunomodulatory potency as much as with their lipid-lowering capacity (such as statins and salicylates) (41).

Given the shared pathophysiology, it is not surprising that individuals with one cardiovascular disease risk factor often have multiple others—an observation that forms the basis for metabolic syndrome.

Insulin Resistance as an Adaptive Trait

Because inflammatory insulin resistance underpins much of the overt pathology associated with obesity and excess caloric intake, we have adopted a biased view of this physiology as a maladaptive response to overfeeding. Although the clinical consequences of obesity are undoubtedly grim, three lines of evidence call into question the current view of insulin resistance as an injurious response to mounting adiposity. First, numerous examples exist in which obesity and insulin resistance are divorced, including both lean, insulin-resistant [such as lipodystrophy and mice in which caveolin-1 has been knocked out (42)] and obese, insulin-sensitive states [such as mice in which *Fabp4* has been knocked out (43) and cold-adapted mammals (44)]. Indeed, the relationship between insulin resistance and obesity in humans is similarly disjointed (45), with many obese individuals exhibiting better insulin sensitivity than expected for their adiposity (46). Moreover, many of the most widely used

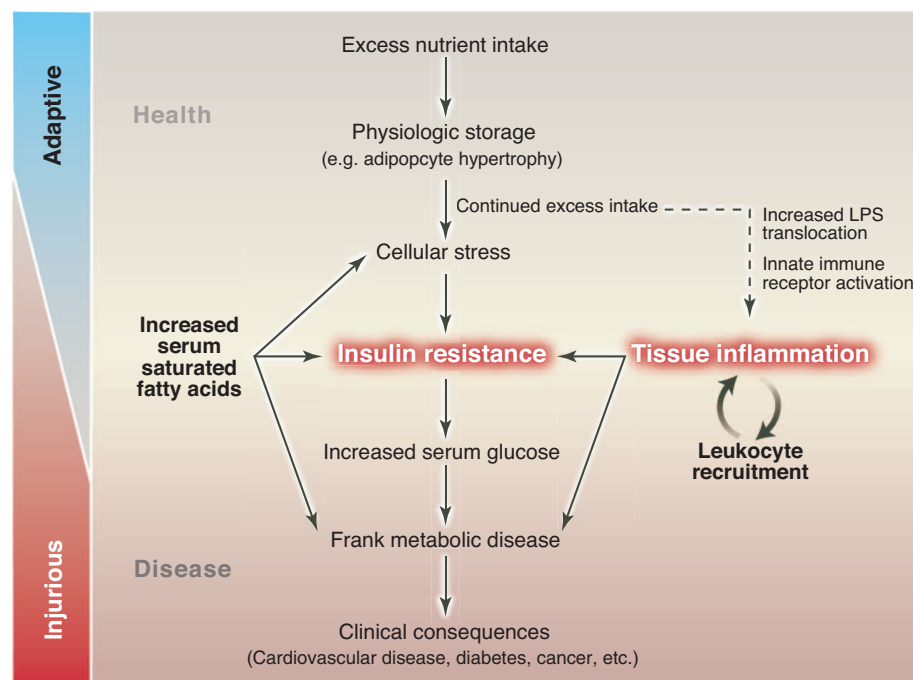


Fig. 2. Inflammation and insulin resistance are central to obesity-induced metabolic disease. Under conditions of acute intake-expenditure imbalance, metabolic tissues store excess nutrients for future use. With chronic imbalance, physiologic storage capacity is exceeded, activating cellular stress signaling pathways that attempt to stem further nutrient influx by inhibiting insulin signaling and promoting inflammation. In adaptive obesity, such as is seen in hibernators, nutrient excess is time-limited, with eventual resumption of physiologic normality before tissue damage can occur. In obesity-induced metabolic disease, however, continued nutrient imbalance drives this process forward, leading to chronic inflammation and insulin resistance and, ultimately, to diabetes, cardiovascular disease, and other overtly pathologic consequences.

insulin-sensitizing pharmaceuticals are associated with an increase in adiposity rather than a decrease [for example, thiazolidinedione treatment (47)].

Second, insulin resistance develops as an evolutionarily conserved adaptive response in specific physiologic contexts unassociated with obesity. For example, both infection and pregnancy require organisms to reserve priority nutrient access for an emerging metabolic requirement—immune system activation and fetal development, respectively, in this instance. Organisms meet these requirements by decreasing systemic insulin sensitivity (developing insulin resistance), decreasing nutrient uptake by nonpriority tissues and reserving glucose for priority cells. Indeed, the resulting adaptive physiology in both situations closely resembles that which develops in the context of obesity (15, 48).

Lastly, extreme diet-induced obesity, complete with systemic insulin resistance, hyperinsulinemia, and hyperlipidemia, is observed as

an evolutionarily conserved, adaptive, and entirely pathology-free response in mammalian hibernators (44). These animals circannually engage in post-reproduction periods of overfeeding and rapidly eat themselves into what in humans would be morbid obesity; some *Zapus* species, for example, enter hibernation with fat comprising more than 80% of their body weight (for comparison, the threshold for human obesity is ~25% in males and ~32% in females) (49). For most hibernators, autumnal obesity is accompanied by physiologic hallmarks of type 2 diabetes and metabolic syndrome, including decreased insulin sensitivity in primary target tissues and substantial elevations in serum insulin, triglyceride, and both total and LDL cholesterol levels (44, 50). Although its exact role is unclear, insulin resistance may function in this context as both a sensor of nutrient stores and as an instructive signal for tissues to switch from glucose to fatty-acid metabolism in preparation for hibernation.

Despite meeting clinical criteria for type 2 diabetes and metabolic syndrome, hibernators demonstrate no pathologic consequences of their brief bout with obesity; after shedding their extra fat during the winter fast, animals are able to immediately enter into the reproductive cycle. Nor are the ill effects of circannual obesity transient; in one Swedish study, atherosclerotic lesions were entirely absent from the major vessels of obese, insulin-resistant hibernators despite marked hyperlipidemia (50). Similarly, obese hibernators fail to develop the smoldering inflammation that characterizes human obesity despite similar metabolic parameters (44, 51). The absence of pathology despite remarkably similar metabolic states suggests that transient obesity and insulin resistance are not necessarily pathologic and may in fact be part of an adaptive, evolutionarily conserved response to excess nutrient storage.

Pregnancy-, sepsis-, and hibernation-associated insulin resistance demonstrate that transient inhibition of insulin signaling can be advantageous in certain contexts. Furthermore, the conservation of these adaptations between organisms as diverse as flies and humans demonstrates that the capacity for insulin resistance is sufficiently advantageous to be conserved through millions of years of evolutionary divergence. This observation, however, is hardly surprising given the evolutionary primacy of infection, starvation, predation, and, above all, reproduction. Indeed, insulin resistance confers evolutionary advantages and enhances organismal fitness in each of these categories: fueling immune function to combat infection, switching hibernator metabolic substrate preference from glucose to lipids to avoid starvation, and reserving metabolic resources for fetal development to optimize reproduction. Insulin resistance is even likely advantageous in predation-driven selection because the “fight-or-flight” response involves antagonism of insulin signaling by the stress-responsive hormones, catecholamines and glucocorticoids (52). In this context, acute inhibition of insulin’s anabolic actions mobilizes stored nutrients to fuel a heightened state of arousal and combat the threat of predation. The pleiotropy and evolutionary importance of variable insulin sensitivity may then explain the diversity of metabolic, inflammatory, hormonal, dietary, and behavioral pathways that influence insulin signaling (Fig. 1).

Many of these conserved and evolutionarily important pathways are active in obesity-induced metabolic disease, and although much of the resulting physiology appears pathologic, insulin resistance’s full effect remains unclear. For example, one of the consequences of insulin resistance in obesity is to limit further nutrient uptake by overloaded cells. Certainly, nutrient toxicity has consequences worth avoiding, which include necrosis of engorged adipocytes found in obese adipose tissue (6). Without insulin resistance to limit further nutrient uptake, this fate may well extend

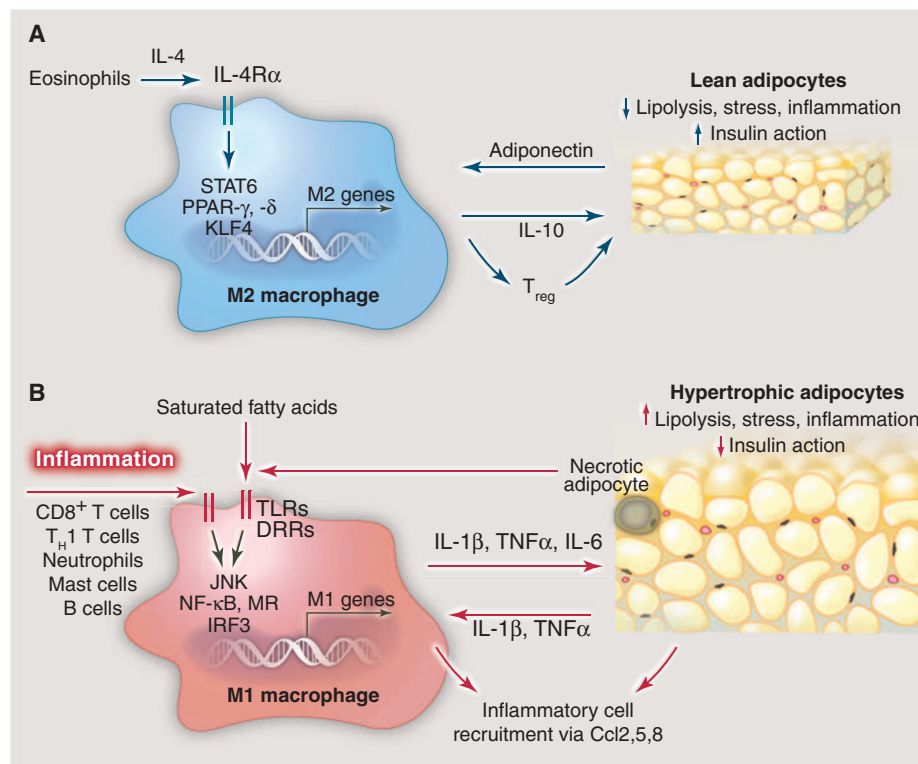


Fig. 3. Lean and obese adipose tissues are associated with distinct macrophage phenotypes (Box 1). In lean adipose tissue (**A**), eosinophil-derived IL-4 supports alternatively activated M2 macrophages characterized by production of tolerogenic cytokines such as IL-10 and minimal production of inflammatory mediators. This phenotype establishes a tolerogenic immune environment and directly promotes adipocyte insulin sensitivity. In turn, lean adipocytes produce adiponectin, which collaborates with IL-4 signaling to enhance alternative M2 macrophage activation. In obese adipose tissue (**B**), inflammatory M1 macrophages, activated by the stigmata of chronic nutrient excess, produce proinflammatory cytokines and chemokines that exacerbate adipocyte insulin resistance, enhance cellular stress, and recruit additional leukocytes. Adipocytes, in turn, also secrete inflammatory cytokines and saturated fatty acids that, along with signals from necrotic cells, reinforce the inflammatory environment. STAT, signal transducer and activator of transcription; KLF, Kruppel-like factor; TLRs, Toll-like receptors; DRRs, Danger recognition receptors; MR, mineralocorticoid receptor; IRF, interferon regulatory factor.

to other adipocytes, decreasing the storage pool available for excess nutrients and establishing a vicious cycle in which fewer and fewer cells are available to shoulder already overwhelming metabolic burdens. After the adipose tissue depots collapse, skeletal myocyte and hepatocyte depots would similarly fail, followed closely by nonprofessional nutrient-storage tissues. Organisms would be able to quite literally eat themselves to death.

Appreciation of this possibility has led some to reconsider the therapeutic potential of insulin-sensitizing treatments as a core approach to obesity-associated metabolic disease (53); targeting a potentially adaptive response to overfeeding—although moderately effective in reducing its unfortunate long-term consequences—is unlikely to effectively treat the underlying physiologic defect. Rather, effective therapies are more likely to target the fundamental energetic imbalance that underpins the entire state (54).

Adaptive Metabolic Leukocyte Activation

Careful study of diet-induced obesity has identified chronic leukocyte-mediated low-grade inflammation within professional metabolic tissues as the characteristic pathophysiology of metabolic syndrome (11, 15). This focus on obesity, however, has limited our understanding of leukocyte activation to its role in promoting metabolic disease. Nonetheless, leukocytes are normally present in metabolic tissues, where they perform nonredundant, supportive functions (26). In lean WAT, for example, eosinophil-derived IL-4 drives production of IL-10 and other mediators by macrophages (Fig. 3). This phenotype is critical for maintenance of both adipocyte insulin sensitivity [IL-10 directly potentiates insulin signaling in adipocytes (16)] and the general anti-inflammatory timbre of the WAT microenvironment (both directly and through support of adipose tissue resident T_{reg} cells) (15). Congruent with these observations, disruption of IL-4 production or signaling in adipose tissue macrophages results in adipocyte dysfunction, insulin resistance, and metabolic disease (17, 23, 27–29). In contrast, augmentation of IL-4 signaling blunts the deleterious effects of high-fat-diet challenge (23, 55).

Alternatively activated M2 macrophages are also an indispensable component of the nonshivering thermogenic response of brown adipose tissue (BAT), the sole dedicated thermogenic tissue in mammals (56). Cold exposure elicits this response via hypothalamic stimulation of BAT-innervating efferents of the sympathetic nervous system that, in turn, activate brown adipocytes by releasing catecholamines. Once activated, brown adipocytes oxidize fatty acids and dissipate the resulting mitochondrial proton gradient via uncoupling protein-1, liberating heat (56). Alternative M2 macrophages form an indispensable component of this adrenergic synapse, accounting for ~50% of the total catecholamine content of cold-

stimulated brown and white adipose tissues (56). In response to cold exposure, alternative M2 macrophages produce catecholamines, which together with sympathetic efferents induce the thermogenic program in brown adipocytes while simultaneously inducing lipolysis in white adipocytes (56). Animals lacking alternative M2 macrophages are thus unable to mount an effective thermogenic response or mobilize the fatty acids necessary to support it.

Even within the context of obesity-induced metabolic disease, leukocyte activation can be adaptive. For example, WAT infiltration by inflammatory M1 macrophages is a well-known, necessary component of metabolic disease; however, augmentation of this leukocyte population might be necessary for certain adaptive roles as well. Infiltration of $Ly6c^{Hi}$ monocytes/M1-biased macrophages in early obesity seems to be driven by the necrosis/apoptosis of hypertrophic adipocytes (6). Although surrounding parenchymal cells can clear apoptotic debris in most organs, adipocyte death yields large lipid droplets, whose uncontrolled lipolysis can be toxic to neighboring cells. Thus, in obese WAT, newly recruited M1 macrophages would encapsulate and sequester the lipid droplet and eventually eliminate it (6). Without professional phagocyte intervention, these adipocyte corpses would presumably persist, releasing necrotic cellular debris and free lipids to the detriment of surrounding tissue.

Although their intervention is undoubtedly necessary, these phagocytic macrophages demonstrate a M1 bias—presumably in response to necrotic debris—and are thought to be a major source of inflammatory cytokines in obese adipose tissue (16). Disposal of apoptotic cell corpses, however, is not necessarily associated with an inflammatory phenotype (57). Indeed, clearance of apoptotic cells potently suppresses macrophage activation and is strongly associated with a regulatory phenotype not entirely dissimilar from that of lean adipose tissue-associated macrophages. Therapeutic manipulation of the manner in which macrophages dispose of dying adipocytes may thus remove a major proinflammatory influence without compromising necessary function.

Integrating Tissue Architecture with Function

The work in obesity, adipose tissue homeostasis, and thermogenesis suggests a close, functional integration of adipocytes and tissue-resident leukocytes in both BAT and WAT. Indeed, the numeric and spatial distribution of these cells within the tissue suggests that this would be the case (58). At baseline, adipose tissue-resident leukocyte number and distribution vary little between individuals and are maintained even across species, whereas their depletion results in rapid and precise restoration of the original leukocyte complement without changes in representation/distribution or encroachment by other populations (26). Some adipose tissue-resident leuko-

cytes even display distinct, tissue-specific features that distinguish them from cells of similar lineage present elsewhere in the body. For example, WAT-resident T_{reg} cells demonstrate a different transcriptional profile characterized by the expression of genes more characteristically associated with neighboring adipocytes, including peroxisome proliferator-activated receptor γ (PPAR γ), the “master regulator” of adipogenesis (59). These data suggest that resident leukocytes may acquire specific features in support of functional roles particular to the tissue in which they reside.

Despite our focus on it thus far, adipose tissue is not singular in its incorporation of leukocytes. Most tissues, in fact, demonstrate orderly complements of resident leukocytes, suggesting that perhaps tissue-resident leukocytes play integrated roles elsewhere as well. Indeed, like adipose tissue-associated macrophages, Kupffer cells of the liver, sinusoidal macrophages of the spleen, microglia of the brain, and alveolar macrophages of the lung are all phenotypically distinct populations with characteristic gene expression patterns and spatial distributions (60). Although there are well-described functional specializations underlying these phenotypic differences in many tissues [for example, senescent red blood cell clearance by splenic red pulp macrophages (60) and neural synapse pruning by microglia (61)], the roles of resident leukocytes remain unexplored in others.

Conclusions

The recognition of obesity as a primary source of human disease has engendered fierce interest in metabolic dysfunction and identified inflammatory insulin resistance as its central pathophysiology. Our focus in the matter, however, largely has been on the study of artificial metabolic extremes—such as high-fat-diet challenge, lipid infusion, and monogenic models. These approaches and the simplifications they circumscribe yield valuable insight into human disease; however, they are limited in their ability to describe the complex, nonextreme forms of obesity-induced metabolic disease that dominate the clinical landscape. For example, a genetically defined caged rodent fed mounds of sugared milk fat and lard over a few months—the basic experimental model of diet-induced obesity—approximates, but will never faithfully recapitulate, an obese human. Our challenge then is to place experimental observations in a more nuanced, relevant context.

First, observations from reductionist disease models such as high-fat feeding must be compared with the disease itself—with clinical observation and intervention. For example, identification of leptin as a potent regulator of feeding behavior by using the *ob/ob* mouse was popularly hailed as an “obesity cure” and was only placed in proper context by subsequent clinical characterization of obesity-related leptin resistance (53). This argument implies that the current popularity of genetic intervention (for example, gene knock-

Box 1. Macrophages

Macrophages are innate immune cells resident in every tissue in the body, where they participate in a variety of homeostatic functions in addition to host defense. These cells exhibit remarkable functional plasticity and versatility; however, when activated, their phenotypes tend to cluster around two general activation programs. M1, or classical, activation is an inflammatory phenotype characterized by robust expression of proinflammatory cytokines (such as TNF- α , IL-1 β , and IL-6), type 1–biasing cytokines (such as IL-12), and reactive nitrogen species (such as nitric oxide). M1 macrophages comprise the primary source for inflammatory cytokines in obese adipose tissue and coordinate inflammatory insulin resistance. The IL-4/IL-13–driven M2, or alternative, activation is a tolerogenic phenotype associated with anti-inflammatory cytokines (such as IL-10 and transforming growth factor- β), anti-parasitic responses (such as Ym-1, dectin-1, and eotaxin), and anabolic functions (such as angiogenesis, fibrosis, and extracellular matrix remodeling). In contrast to M1 macrophages, these cells are critical for the maintenance of adipocyte insulin signaling and anchor the tolerogenic environment of lean adipose tissue. Although all macrophages generally express F4/80 and CD11b, M1 macrophages in adipose tissue can be distinguished *in vivo* by their CD11c^{Hi}Nos2⁺TNF- α ⁺ immunophenotype, whereas alternative M2 cells are CD206⁺CD301⁺Arg1⁺. Although these basic activation programs represent general phenotypic responses to pathogens, tissue macrophages do not always rigidly adhere to these expression profiles.

outs, floxed alleles, and gain- or loss-of-function mutants) must be tempered by careful search for similar aberrations in human cohorts, as exemplified by the recent studies on Gpr120 (62). Moreover, this comparison can also be exploited in the reverse direction. Because of the “trial and error” approach of much clinical research, both therapeutic successes and failures are often instructive, as demonstrated by both the relative success of surgical approaches and failure of pharmaceuticals in the treatment of obesity (63).

Second, disease models and clinical observations must be compared with similar models and clinical observations of health. For example, leukocyte activation is a central pathophysiology in diet-induced obesity; however, the protective effects of WAT- and liver-associated macrophages and WAT-associated T cells in lean, healthy rodents clearly demonstrate that leukocyte activation is context-dependent and explain the otherwise paradoxical exacerbation of metabolic pathology that accompanies complete loss of these lineages. Indeed, these observations have suggested novel therapeutic avenues, including pharmacologic skewing of macrophages and lymphocytes toward regulatory phenotypes. In addition, a number of inflammatory pathways that regulate energy expenditure and insulin resistance—such as IKK ϵ , PKR, and Gpr120—might be suitable for therapeutic targeting of obesity-associated metabolic disease (10, 62, 64, 65).

Last, disease models and clinical observations must be compared with physiologically similar but pathologically distinct situations. The negative effects of insulin resistance, for instance, are much less clear in the context of pregnancy and infection, whereas the pathology-free “metabolic syndrome” of obese hibernators suggests that our current understanding of causality in human diet-induced obesity is incomplete. Although studies focusing on this particular context are rare, we have already discussed three biological scenarios—

pregnancy, infection, and hibernation—in which aspects of obesity-induced metabolic disease manifest and regress. Careful study of how organisms reestablish metabolic normality after these events may provide insight into how we might reestablish the same in obesity-induced metabolic disease.

Our current knowledge of metabolic biology and the basic mechanisms of obesity-induced metabolic disease is impressive. The remarkable progress in this field, however, has largely failed to translate into meaningful therapeutic advances in our struggle with obesity and metabolic disease, in part because of a failure to place empiric studies in the proper clinical, physiological, and biological context. The challenge going forward, then, is to provide that context for the wealth of information available in order to identify root pathophysiologies and to appropriately target emerging therapeutics.

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Quantum Back-Action of an Individual Variable-Strength Measurement

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Measuring a quantum system can randomly perturb its state. The strength and nature of this back-action depend on the quantity that is measured. In a partial measurement performed by an ideal apparatus, quantum physics predicts that the system remains in a pure state whose evolution can be tracked perfectly from the measurement record. We demonstrated this property using a superconducting qubit dispersively coupled to a cavity traversed by a microwave signal. The back-action on the qubit state of a single measurement of both signal quadratures was observed and shown to produce a stochastic operation whose action is determined by the measurement result. This accurate monitoring of a qubit state is an essential prerequisite for measurement-based feedback control of quantum systems.

Although the evolution (“state collapse”) of a quantum system subject to an infinitely strong (i.e., projective) quantum nondemolition (QND) measurement is textbook physics, the subtlety and utility of finite strength (i.e., partial) measurement phenomena are neither widely appreciated nor commonly verified experimentally. Standard quantum measurement theory puts forward the principle that observing a system induces a decoherent evolution proportional to the measurement strength (1–5). Thus, partial measurement is often associated with partial decoherence of the state of a quantum system. However, this measurement-induced degradation occurs only if the measurement is inefficient informationally—that is, if only a portion of the measurement’s information content is available to the observer for use in reconstructing the new state of the system.

If, instead, the measurement apparatus is entirely efficient, the new state of the quantum system can be perfectly reconstructed. This outcome-dependent revision of the system’s imposed initial conditions constitutes a fundamental quantum effect called measurement back-action (2, 6–8). Although the system’s evolution under measurement is erratic (hence, the measurement outcome cannot be predicted in advance), the measurement record faithfully reports the perturbation of the system after the fact.

We use the powerful, combined qubit-cavity architecture, circuit quantum electrodynamics (cQED) (9, 10), which allows for rapid, repeated quantum nondemolition (QND) (11, 12) superconducting qubit measurement (13–18). The

cavity output is monitored in real time by using a phase-preserving amplifier working near the quantum limit, where the noise is only caused by the fundamental quantum fluctuations of the electrodynamic vacuum (19). The decision to read out our qubit by using coherent states of the resonator has two important consequences. First, the outcomes of a partial measurement form a quasi-continuum, unlike the set of discrete answers obtained from a projective measurement. Second, measuring both quadratures of the signal leads to two-dimensional (2D) diffusion of the direction of the qubit effective spin. We show that the choice of measurement apparatus and of measurement strength both affect the evolution of a quantum system, but neither results in degradation of the system’s state if the measurement is informationally efficient. Such precise knowledge of the measurement back-action is a necessary prerequisite for general feedback control of quantum systems.

Our superconducting qubit is a transmon (20), consisting of two Josephson junctions in a closed loop, shunted by a capacitor to form an anharmonic oscillator. The two lowest energy states, ($|g\rangle$ and $|e\rangle$), are the logical states of the qubit. The qubit is dispersively coupled to a compact resonator, which is further asymmetrically coupled to input and output transmission lines (Fig. 1, B and C), determining the resonator bandwidth ($\kappa/2\pi = 5.8$ MHz). To measure a qubit prepared in initial state $|\psi\rangle = c_g|g\rangle + c_e|e\rangle$, a microwave pulse of duration $T_m \gg 1/\kappa$ is applied to the resonator. The state-dependent shift of the resonator frequency ($\chi/2\pi = 5.4$ MHz) results in an entangled state of the qubit and pulse $|\Psi\rangle = c_g|g\rangle \otimes |\alpha_g\rangle + c_e|e\rangle \otimes |\alpha_e\rangle$, where $|\alpha_{g,e}\rangle$ refers to the coherent state after traversing the resonator.

Amplification is required to convert the pointer state $|\Psi\rangle$ into a macroscopic signal that can be processed and recorded with standard instrumentation. In our case, the pulse, having traversed the resonator, is amplified by using a linear, phase-preserving amplifier with gain G , which can be

seen as multiplying the average photon number in $|\alpha_{g,e}\rangle$ (Fig. 1B). For dynamical range considerations, our amplifier, called the Josephson parametric converter (JPC) (21–24) is operated in this experiment with a gain $G = 12.5$ dB and bandwidth of 6 MHz, adding close to the minimum amount of noise allowed by quantum mechanics. The added quantum fluctuations are due to a second, “idler,” input (19). A measurement of both quadratures of the output mode results in an outcome, denoted (I_m, Q_m) , which is then used to determine the new state of the qubit after measurement (Fig. 1A). As has been shown in (8), and detailed in the supplementary materials, this outcome contains all information necessary to perfectly reconstruct the new state of the qubit. Remarkably, the additional quantum fluctuations introduced during amplification enter in the measurement back-action on the qubit without impairing our knowledge of it.

We first demonstrate projective qubit readout by strongly measuring the qubit using an 8- μ s pulse with the drive power set so that the average number of photons in the resonator during the pulse was $\bar{n} = 5$ (Fig. 2). Selected individual measurement records for the qubit are shown in Fig. 2B. The data are digitized with a sampling time of 20 ns and smoothed with a binomial filter with $T_m = 240$ ns width, which corresponds to eight cavity lifetimes, and scaled by the experimentally determined standard deviation (σ). The highlighted trace shows clear quantum jumps in the qubit state, which are identified by vertical black dotted lines indicating 4σ deviations from the current qubit state. The 8% equilibrium qubit excited-state population is consistent with other measurements of superconducting qubits (25). By counting the number of up and down transitions in 25,000 traces with no qubit excitation pulse, we calculate $T_1 \leq 3.1$ μ s. Although we fail to resolve pairs of transitions separated by much less than our filter time constant, this method for estimating T_1 yields a value in good agreement with the value $T_1 = 2.8$ μ s calculated from fitting an exponential to the averaged trajectory of all traces. Further, the average qubit polarization did not vary over 8 μ s of continuous measurement, nor did T_1 diminish with larger readout amplitude up to $\bar{n} \approx 15$, which demonstrates the QND nature of our readout.

Histograms of the scaled I_m component of the outcome for the first 240 ns of measurement after a qubit rotation by $\theta = 0, \pi/2, \pi$ are shown in Fig. 2C. The ground and excited distributions are separated by 4.8σ , which corresponds to a measurement fidelity of 98% when $I_m = 0$ is used as the discrimination threshold. We emphasize that the discreteness of the z measurement of the transmon circuit, illustrated by the bimodality of the histogram, is here due only to the quantum nature of the circuit and not to any nonlinearity of the readout. Thus, this measurement of a continuous, unbounded pointer state is exactly equivalent to the Stern-Gerlach experi-

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ment. These strong, high-fidelity measurements allow us to perform precise tomography and to prepare the qubit in a known state by measurement. We next use these tools to quantify measurement back-action of partial measurement on the qubit state.

The qubit evolution due to partial measurement can be precisely calculated from the complete measurement record using the quantum trajectory approach (6, 7), but this is computationally intensive. Instead, we calculate the back-action from the average output over the time T_m as in (8). Provided that the measurement time is short compared with the qubit coherence times T_1 and T_2 , and long compared with the cavity lifetime and amplifier response time, this approach allows the qubit to be tracked without degradation. In this experiment, $T_m = 240$ ns, which is shorter than $T_1 = 2.8$ μ s and $T_{2R} = 0.7$ to 2.0 μ s, and much longer than the cavity lifetime and JPC response time of 30 ns.

Assuming the qubit is initially polarized along the $+y$ axis, we calculate the final qubit Bloch vector (x_f, y_f, z_f) as a function of measurement

outcome (I_m, Q_m) (see detailed derivation in the supplementary materials) to be

$$\begin{aligned} x_f^\eta(I_m, Q_m) &= \text{sech}\left(\frac{I_m \bar{T}_m}{\sigma^2}\right) \\ &\times \sin\left[\frac{Q_m \bar{T}_m}{\sigma^2} + \frac{\bar{Q}_m \bar{T}_m}{\sigma^2} \left(\frac{1-\eta}{\eta}\right)\right] \\ &\times e^{-\frac{\bar{T}_m^2}{\sigma^2} \left(\frac{1-\eta}{\eta}\right)}, \\ y_f^\eta(I_m, Q_m) &= \text{sech}\left(\frac{I_m \bar{T}_m}{\sigma^2}\right) \\ &\times \cos\left[\frac{Q_m \bar{T}_m}{\sigma^2} + \frac{\bar{Q}_m \bar{T}_m}{\sigma^2} \left(\frac{1-\eta}{\eta}\right)\right] \\ &\times e^{-\frac{\bar{T}_m^2}{\sigma^2} \left(\frac{1-\eta}{\eta}\right)}, \\ z_f^\eta(I_m) &= \tanh\left(\frac{I_m \bar{T}_m}{\sigma^2}\right) \end{aligned} \quad (1)$$

where $\bar{T}_m$ and $\bar{Q}_m$ and σ define the center and standard deviation of the outcome distributions,

and η is the quantum efficiency of the amplification chain (Fig. 1A). In this theory, we neglect the effect of qubit decoherence and losses before amplification. In the limit of a perfectly efficient amplification ($\eta = 1$), we see that the length of the Bloch vector is unity, irrespective of outcome. The parameter $\bar{T}_m/\sigma$ can be identified as the apparent measurement strength because the measurement becomes more strongly projective as $\bar{T}_m/\sigma$ increases. It is given in terms of experimental parameters as $\bar{T}_m/\sigma = \sqrt{2\pi\eta\kappa T_m \sin(\vartheta/2)}$, where $\vartheta = 2 \arctan \chi/\kappa$.

The pulse sequence for determining measurement back-action is shown in Fig. 3A. We first strongly read out the qubit with a 240 ns, $\bar{n} = 5$ pulse and record the outcome, which will be used to prepare the qubit in the ground state by post-selection. Then, the qubit is rotated to the $+y$ axis and measured with a variable measurement strength ($T_m = 240$ ns), and the outcome (I_m, Q_m) is recorded. The final, tomography, phase measures the x , y , or z component of the qubit Bloch vector with a strong ($\bar{n} = 5$, $T_m = 240$ ns) measurement pulse. To compensate for the finite

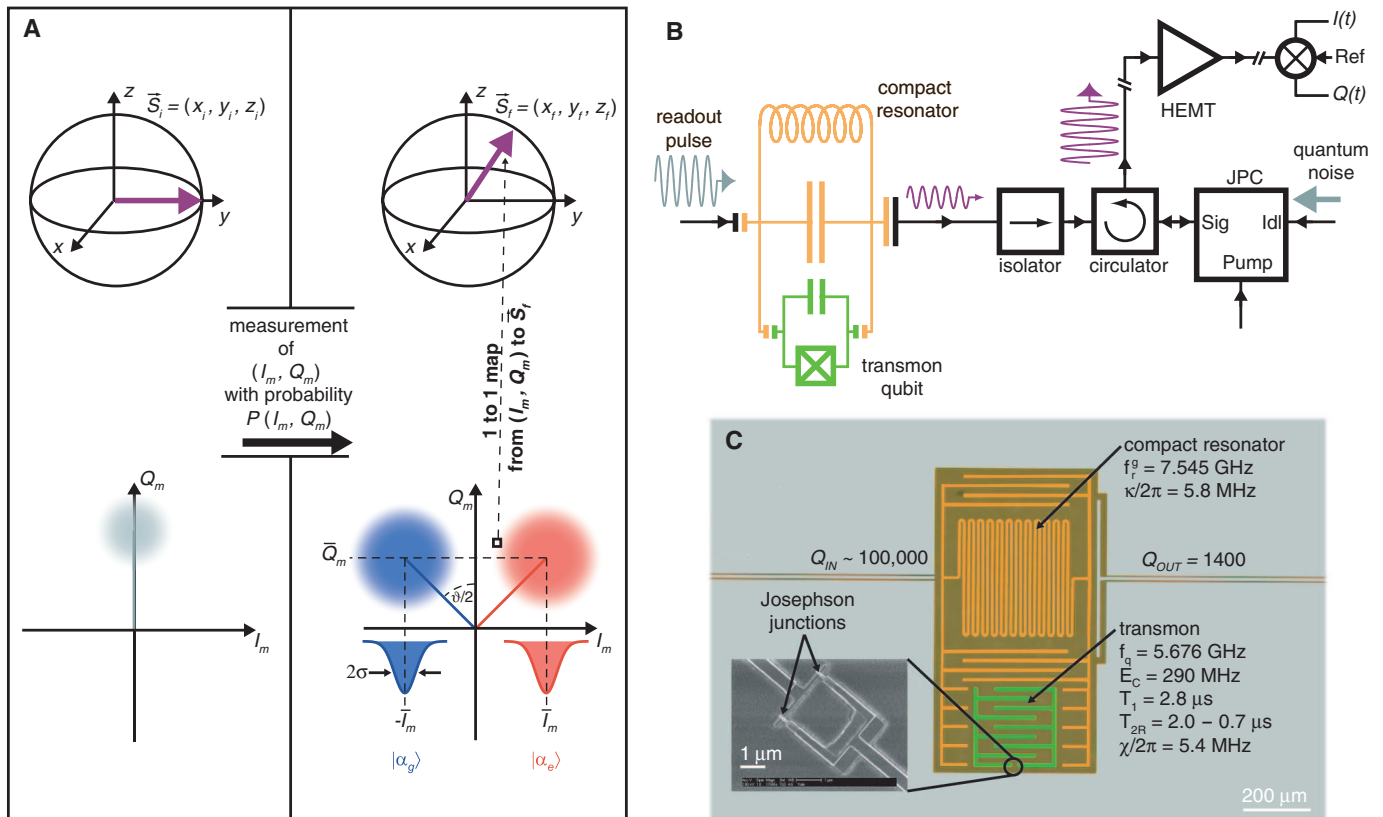


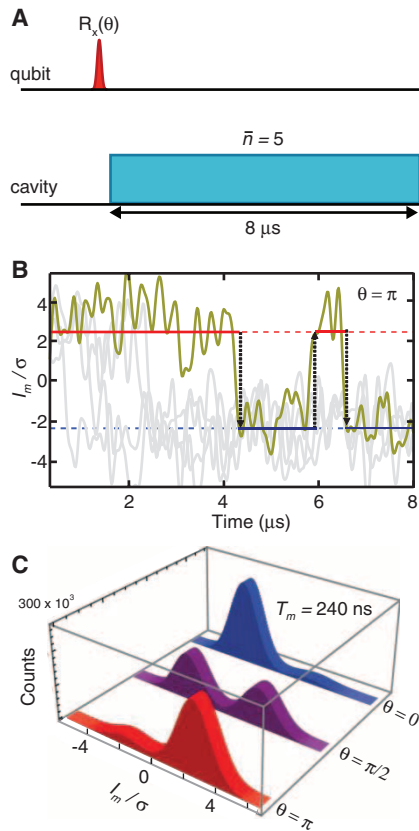
Fig. 1. (A) Bloch sphere representation of the effect on the qubit state of a phase-preserving measurement in a cQED architecture. After a measurement with outcome (I_m, Q_m) , the qubit will be found in a final state $\vec{S}_f = (x_f, y_f, z_f)$, with I_m encoding information on the projection of the qubit state along z and corresponding back-action and Q_m encoding the other component of the back-action, which is parallel to $\hat{z} \times \vec{S}_i$. The measurement outcomes are Gaussian distributed, with $\bar{T}_m^2 + \bar{Q}_m^2 = \bar{n}\kappa T_m$. (B) Schematic of experiment mounted to the base plate of a dilution refrigerator. Readout pulses are transmitted through the strongly coupled port of the resonator, via an iso-

lator and circulator, to the signal port (Sig) of a JPC. The idler port (Idl) is terminated in a 50 Ω load. The amplified signal output is routed via the circulator and further isolators (not shown) to a high electron mobility transistor (HEMT) amplifier operated at 4 K, and subsequently demodulated and digitized at room temperature. (C) False-color photograph of the transmon qubit in compact resonator with qubit and resonator parameters. Inset is a scanning electron micrograph of the center of the transmon showing a loop of two Al/AlOx/Al junctions that form an effective junction tunable by an external magnetic field.

readout strength and qubit temperature, trials with outcomes $|I_m/\sigma| < 1.5$ (corresponding to state purity $< 99\%$) for the first and third measurements are discarded, as well as outcomes for the first measurement with the qubit in $|e\rangle$. To quantify the measurement back-action for a given measurement outcome (I_m, Q_m) , the average final qubit

Bloch vector, conditioned by the measurement outcome (I_m, Q_m) , $(\langle X \rangle_c, \langle Y \rangle_c, \langle Z \rangle_c)$, is calculated versus outcome using the results of the tomography phase. These conditional maps of $\langle X \rangle_c, \langle Y \rangle_c, \langle Z \rangle_c$ were constructed using 201 by 201 bins in the plane of scaled measurement outcomes $(I_m/\sigma, Q_m/\sigma)$.

Fig. 2. (A) Pulse sequence for strong measurement. An initial qubit rotation $R_x(\theta)$ of θ radians about the x axis is followed by an $8\text{-}\mu\text{s}$ readout pulse with drive power such that $\bar{n} = 5$. **(B)** Individual measurement records. The data are smoothed with a binomial filter with a $T_m = 240$ ns time constant and scaled by the experimentally determined standard deviation (σ). Black dotted lines indicate 4σ deviation events. The qubit is initially measured to be in the excited state, and quantum jumps between excited and ground states are clearly resolved. The center of the ground- and excited-state distributions are represented as horizontal dotted lines. **(C)** Histograms of the initial 240-ns record of the readout pulse along I_m axis, for $\theta = 0, \pi/2, \pi$. Finite qubit temperature and T_1 decay during readout are visible as population in the undesired qubit state.



Results for four measurement strengths increasing by decades from $\bar{n} = 5 \times 10^{-3}$ to 5 are shown in Fig. 3B (see movie S1 of histograms and tomograms for all measurement strengths). The left column shows a 2D histogram of all scaled measurement outcomes recorded during the variable-strength readout pulse. At weak measurement strength, the ground- (left) and excited- (right) state distributions overlap almost completely. Their separation grows with increasing strength until they are well separated at $\bar{n} = 5$, which corresponds to the strong projective measurement shown in Fig. 1A. The rightmost columns show $\langle X \rangle_c, \langle Y \rangle_c, \langle Z \rangle_c$ versus the associated $(I_m/\sigma, Q_m/\sigma)$ bin. At weak measurement strength ($\bar{n} \ll 1$), the qubit state is only slightly perturbed, with all measurement outcomes corresponding to Bloch vectors pointing nearly along the $+y$ (initial) axis. However, gradients in $\langle X \rangle_c$ along the Q_m axis and $\langle Z \rangle_c$ along the I_m axis are visible, demonstrating the outcome-dependent back-action of the measurement on the qubit state. As the measurement strength increases, so does the back-action, as seen in the increase of the gradients in the $\langle X \rangle_c$ and $\langle Z \rangle_c$ maps (see fig. S2). When the measurement becomes strong, the qubit is projected to $+z$ for positive I_m ($-z$ for negative I_m), whereas $\langle X \rangle$ and $\langle Y \rangle$ go unconditionally to zero, as expected.

One of the key predictions of finite-strength measurement theory is that the statistics of the measurement process, in particular the apparent measurement strength in the I-quadrature (which can be determined experimentally from the statistics of the measurement outcomes), are sufficient to infer z_f for any apparent measurement strength or outcome (see Eq. 1). For weak measurement, where the back-action is symmetric along both x and z , the apparent measurement strength deter-

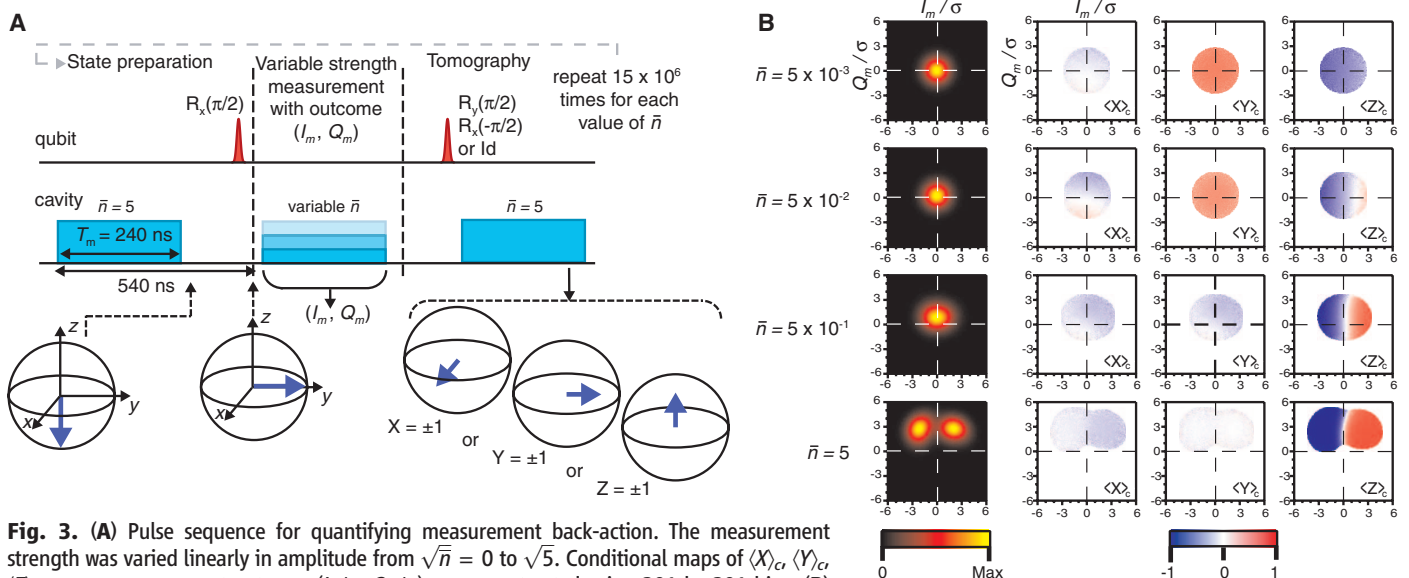


Fig. 3. (A) Pulse sequence for quantifying measurement back-action. The measurement strength was varied linearly in amplitude from $\sqrt{\bar{n}} = 0$ to $\sqrt{5}$. Conditional maps of $\langle X \rangle_c, \langle Y \rangle_c, \langle Z \rangle_c$ versus measurement outcome $(I_m/\sigma, Q_m/\sigma)$ were constructed using 201 by 201 bins. **(B)** Results are shown increasing by decades from $\bar{n} = 5 \times 10^{-3}$ to 5. The left column shows a 2D histogram of all scaled measurement outcomes recorded during the variable-strength readout pulse. The three rightmost columns are tomograms showing $\langle X \rangle_c, \langle Y \rangle_c, \langle Z \rangle_c$ versus the associated $(I_m/\sigma, Q_m/\sigma)$ bin.

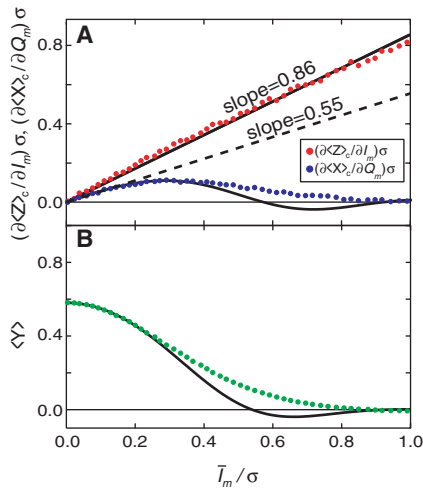


Fig. 4. Correlation between back-action and measurement outcome. **(A)** Experimental data for correlated back-action signal along z , $(\partial\langle Z\rangle/\partial I_m)\sigma$, and along x , $(\partial\langle X\rangle/\partial Q_m)\sigma$, evaluated at $(I_m, Q_m) = 0$, are plotted versus $\bar{T}_m/\sigma$. For weak measurement strength, the slopes at the origin (represented by solid and dashed line, for z and x , respectively) agree with theoretical predictions, including first-order corrections for T_1 and T_2 . The solid curve is the full theoretical expression for the x back-action plotted with $\eta = 0.2$, $\bar{Q}_m = 1.28 \bar{T}_m$, and $\exp(-\tau/T_2) = 0.58$. **(B)** Experimental data for unconditioned $\langle Y \rangle$ versus $\bar{T}_m/\sigma$. The data show the expected measurement-induced dephasing when the measurement outcome is not used to condition the perturbed qubit state. The dephasing rate is proportional to $(\bar{T}_m/\sigma)^2$, resulting in the apparent Gaussian dependence of $\langle Y \rangle$ versus $\bar{T}_m/\sigma$. The theoretical expression for $\langle Y \rangle$ versus $\bar{T}_m/\sigma$ with parameters listed above is shown as a solid curve.

mines the amplitude of the x back-action as well (see eq. 14 in the supplementary materials). In Fig. 4A, we quantitatively compare this prediction with our experimental result. The scaling coefficients relating measurement outcome to back-action along z , $(\partial\langle Z\rangle/\partial I_m)\sigma$, and along x , $(\partial\langle X\rangle/\partial Q_m)\sigma$, extracted from the tomograms at $I_m = Q_m = 0$, are plotted versus the apparent measurement strength extracted from the histograms, $\bar{T}_m/\sigma$ (see section 1.4 in the supplementary materials).

Both coefficients, $(\partial\langle Z\rangle/\partial I_m)\sigma$ and $(\partial\langle X\rangle/\partial Q_m)\sigma$, are predicted at $I_m = Q_m = 0$ to be equal to $\bar{T}_m/\sigma$; therefore, the data in Fig. 4A should have unity slope. However, finite T_1 and T_2 acting for a time τ reduce the state purity and the apparent back-action. To first order, the coefficients are modified to $(\partial\langle Z\rangle/\partial I_m)\sigma = (\bar{T}_m/\sigma)e^{-\tau/T_1}$ and $(\partial\langle X\rangle/\partial Q_m)\sigma \cong (\bar{T}_m/\sigma)e^{-\tau/T_2}$ for the z and x back-action, respectively. In our pulse sequence, $\tau \cong 380$ ns, predicting slopes of 0.87 ± 0.09 and 0.58 ± 0.06 for z and x , in excellent agreement with the experimentally determined slopes of 0.86 ± 0.01 and 0.55 ± 0.01 . All further theoretical predictions are modified to reflect the effects of T_1 and T_2 , following the description in eq. 2 in the sup-

plementary materials. The black curve is the full theoretical dependence of $(\partial\langle X\rangle/\partial Q_m)\sigma = \bar{T}_m/\sigma \cos\{\bar{Q}_m \bar{T}_m/\sigma^2[(1-\eta)/\eta]\}e^{-\bar{T}_m^2/\sigma^2(1-\eta)\tau/T_2}e^{-\tau/T_2}$ using $\eta = 0.2$, the lowest value of η we extract from other measurements (see section 1.3 in the supplementary materials). We attribute the discrepancy between theory and data at high measurement strength to environmental dephasing effects due to finite T_2 and losses before the JPC. Additionally, we process the tomography results unconditioned by measurement outcome in Fig. 4B. Theory predicts $\langle Y \rangle = e^{-\bar{T}_m^2/\eta\sigma^2} \cos(\bar{T}_m \bar{Q}_m/\eta\sigma^2) e^{-\tau/T_2}$. This expression evaluated with $\eta = 0.2$ is shown as a black curve with the deviation for stronger measurements attributed to dephasing effects due to losses before amplification.

Similar experiments have studied measurement of the state of a microwave cavity by Rydberg atoms (26) and partial nonlinear measurement of phase qubits (27). Also, phase-sensitive parametric amplification has been used to implement weak measurement-based feedback (18). In our experiment, the ability to perform both weak and strong high-efficiency, QND, linear measurements within a qubit lifetime, coupled with our high-throughput and minimally noisy readout electronics, allows us to acquire 13.5 billion qubit measurements in ~ 28 hours, data that can be compared with complete theoretical predictions of the conditional evolution of quantum states under measurement. They provide strong evidence that the purity of the state would not decrease in the limit of a perfect measurement, even when the signal is processed by a phase-preserving amplifier.

Our experiment illustrates an alternate approach to the description of a quantum measurement. In the case of a qubit, a finite-strength QND measurement can be thought of as a stochastic operation whose action is unpredictable but known to the experimenters after the fact if they have a quantum-noise-limited amplification chain. Any final state is possible, and the type of quantity measured, combined with the measurement strength, determines the probability distribution for different outcomes. This partial (i.e., finite strength) measurement paradigm is not inconsistent with the usual view of projective (i.e., infinite strength) measurement. Rather, projective measurement is the limiting case of the broader class of finite strength measurements.

The finite-strength measurement predictions that we have verified have immediate applicability to proposed schemes for feedback stabilization and error correction of superconducting qubit states. Whereas classical feedback is predicated on the idea that measuring a system does not disturb it, quantum feedback has to make additional corrections to the state of the system to counteract the unavoidable measurement back-action. The measurement back-action that is the subject of this paper thus crucially determines the transformation of the measurement outcome into the optimal correction signal for feedback. Our ability to experimentally quantify the back-action of an arbitrary-strength measurement thus provides a

dress rehearsal for full feedback control of a general quantum system.

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Supplementary Materials

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Materials and Methods
Supplementary Text
Figs. S1 to S3
References
Movie S1

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Strong, Light, Multifunctional Fibers of Carbon Nanotubes with Ultrahigh Conductivity

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Broader applications of carbon nanotubes to real-world problems have largely gone unfulfilled because of difficult material synthesis and laborious processing. We report high-performance multifunctional carbon nanotube (CNT) fibers that combine the specific strength, stiffness, and thermal conductivity of carbon fibers with the specific electrical conductivity of metals. These fibers consist of bulk-grown CNTs and are produced by high-throughput wet spinning, the same process used to produce high-performance industrial fibers. These scalable CNT fibers are positioned for high-value applications, such as aerospace electronics and field emission, and can evolve into engineered materials with broad long-term impact, from consumer electronics to long-range power transmission.

On the molecular level, carbon nanotubes (CNTs) have an outstanding combination of mechanical strength and stiffness, electrical and thermal conductivity, and low density, making them ideal multifunctional materials that combine the best properties of polymers, carbon fibers, and metals (*1*). However, such outstanding properties have remained elusive on a macroscopic scale. Handling CNTs with sufficient length, stiffness, and chemical inertness introduces major challenges in material processing. Here we report lightweight fibers that approach the high specific strength of polymeric and carbon fibers, while also achieving the high specific electrical conductivity of metals and the specific thermal conductivity of graphite fibers.

Two distinct routes have been developed for manufacturing neat CNT fibers (*2*). One route employs a solid-state process wherein CNTs are either directly spun into a fiber from the synthesis reaction zone (*3, 4*) or from a CNT forest grown on a solid substrate (*5*). This approach does not lend itself to the typical easy scale-up of chemical processes, as it combines multiple steps into a single one, limiting the options for process and material optimization. Indeed, solid-state fibers have low packing and poor orien-

tation, and include impurities within their structure (*6*). Despite these shortcomings, solid-state CNT fibers have delivered the best properties so far (*3, 4, 7–9*). The reason for this relative success is the length of the CNTs that constitute these fibers—1 mm or more (*2*). Longer CNTs reduce the number of CNT ends in a fiber, yielding greater strength (*10*) and reducing CNT junctions, which increases electrical and thermal conductivity (*11*). The alternate fiber production route—wet spinning—was the first method for producing CNT fibers (*12*). In this process, premade CNTs are dissolved or dispersed in a fluid, extruded out of a spinneret, and coagulated into a solid fiber by extracting the dispersant. Wet spinning is easily scaled to industrial levels and is indeed the route by which high-performance fibers are manufactured (including ballistic fibers such as Kevlar and Twaron and structural fibers such as Toho Tenax and Thorne carbon fibers) (*13*). Decoupling the synthesis of CNTs from the spinning of the fibers allows the independent optimization of the two steps and enables CNT purification. An important variation of the original wet spinning method was the use of acid as a solvent and standard coagulants like water (*14*), thereby simplifying the original method (*12*) by avoiding surfactants in the CNT dispersion and eliminating polymer from the coagulation bath. So far, wet spinning from acid has yielded the most highly ordered and dense CNT fibers (*14*). Yet, their properties have been disappointing. Inadequate CNT length [$\sim 0.5 \mu\text{m}$ (*14*)] has been the presumed culprit (*2*), and the literature concurs that wet spinning is inappropriate for handling long CNTs. Here we show that exciting properties can be achieved by wet-spinning $\sim 5\text{-}\mu\text{m}$ “short” CNTs into high-performance multifunctional fibers.

High-quality CNTs (figs. S1 to S6) were dissolved (*15*) in chlorosulfonic acid (the only

known CNT solvent) (*16–18*) at a concentration of 2 to 6 weight (wt) % and filtered to remove particles, in order to form a spinnable liquid crystal dope (Fig. 1A and fig. S7). The dope was extruded through a spinneret (65- to 130- μm diameter) into a coagulant (acetone or water) to remove the acid. The forming filament was collected onto a winding drum (Fig. 1, B to E, and movie S1). The linear velocity of the drum was higher than the dope speed at the spinneret exit, to ensure high CNT alignment by continuous stretching and tensioning of the filament. The fibers were further washed in water and dried in an oven at 115°C.

Fibers were tested for mechanical, electrical, and thermal properties. Tensile strength, modulus, and elongation to break (*15*) were determined from tensile break tests on macroscopic (~ 20 mm long) individual filaments cut from large spools (~ 100 to 500 m). Stress was calculated by dividing the applied force by the fiber cross-sectional area determined by scanning electron microscopy (SEM) (fig. S8) and light microscopy (fig. S9). The average tensile strength was 1.0 ± 0.2 GPa (best value 1.3 GPa, fig. S10A), and the average modulus was 120 ± 50 GPa (best value 200 GPa; fig. S10, B and C). The average elongation at break for these fibers was $1.4 \pm 0.5\%$. These same fibers displayed high electrical conductivity [measured by two- and four-point probe on 25-mm single filaments (*15*)], on average 2.9 ± 0.3 MS/m (resistivity of 35 ± 3 microhm cm) at room temperature; doping by iodine (*15*)—a known, stable CNT dopant (*19*)—increased conductivity to 5 ± 0.5 MS/m (resistivity 22 ± 4 microhm cm, best value of 17.5 microhm cm); these values were stable over 1 year in laboratory conditions and also under thermal cycling to 200°C in air for 24 hours. We also measured an average thermal conductivity of 380 ± 15 W/m K on $\sim 1.5\text{-mm}$ -long samples using the 3-omega method (*15, 20*). Iodine doping doubled thermal conductivity (635 W/m K). Such high thermal conductivity remains unchanged after annealing at 600°C, whereas the electrical conductivity drops by an order of magnitude to 0.4 MS/m (resistivity of 240 microhm cm). Fiber density [measured by weighing a 60-m filament with $(9.0 \pm 0.6)\text{-}\mu\text{m}$ diameter] was 1.3 ± 0.1 g/cm³; iodine doping increased it to 1.4 g/cm³, on the basis of a 10% mass increase determined from thermal gravimetric analysis (fig. S11).

These combined properties are remarkable when compared to those of other CNT fibers and high-performance materials (Fig. 2 and table S2); these properties are related to CNT length, alignment, type (including diameter and number of walls), graphitic character, and purity. Tensile strength shows a 10-fold improvement over wet-spun fibers of 0.5- μm CNTs [~ 0.11 GPa (*14*)] and is comparable to the best macroscopic samples of solid-state spun fibers of millimeter-long CNTs [best values of ~ 1.8 GPa assuming a density of 1 g/cm³ (*7*)]. We find that CNT length, aspect ratio, and purity

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are key to strength improvements, because of better CNT-CNT stress transfer and lower defect density; in contrast, CNT orientation, graphitic character, and type are not as critical for attaining high strength, although a higher number of walls lowers the specific strength. The modulus is improved over that of earlier wet-spun 0.5- μm CNT fibers [(14)] and higher than that of the best solid-state CNT fibers [~120 GPa assuming a density of 1 g/cm³ (7)], but lower than the modulus of graphite fibers (GF) (~1 TPa). Modulus is principally affected by orientation, which was already high in earlier studies (14); the increased CNT orientation in our fibers is counterbalanced by the lowered theoretical modulus (~350 GPa) due to larger CNT diameter (21). Electrical conductivity of doped fibers is 10-fold that of wet-spun 0.5- μm CNT fibers and the best continuous,

undoped solid-state fibers [0.5 MS/m (14, 22)]; three to five times better than that of the best doped CNT fibers [~1.3 MS/m (23)] and isolated ropes [~1 MS/m (24, 25)]; and comparable to that of the best solid-state, acid-densified, iodine-doped individual CNT fibrils [5.8 MS/m (26)]. The fiber electrical conductivity did not degrade when kinks were progressively introduced (figs. S12 and S13), indicating that these fibers, unlike copper (27), resist bending fatigue. We find that electrical conductivity is not as sensitive as modulus to CNT alignment and impurities; length is not critical in the tested range of ~3 to ~7 μm (but is likely important in the range 0.5 to 3 μm). The key improvements over earlier fibers are due to the combined effect of CNT length, type, and graphitic character, as also observed recently in CNT films (28). Thermal conductivity appears to follow electrical

conductivity [as in conventional carbon fibers (29)]; it is ~30 times as high as that of wet-spun 0.5- μm CNT fibers [21 W/m K (14)], ~10 times as high as that of the best solid-state CNT fibers [60 W/m K (30)], and ~3 times as high as that of the best magnetically aligned discrete CNT films [200 W/m K (31)].

Figure 2, B and C, compare CNT fibers (from this article and from the literature) to the materials with the best mechanical, electrical, and thermal properties. Our CNT fibers combine the typical specific electrical conductivity of metal wires (copper, silver, and aluminum) with the typical specific strength of high-performance carbon fibers.

To understand the fiber structure-properties relationship, we studied fiber morphology [by high-resolution SEM (HR-SEM) (15)], alignment [by single filament wide angle x-ray diffraction, WAXD (15)], and packing fraction [by high-resolution

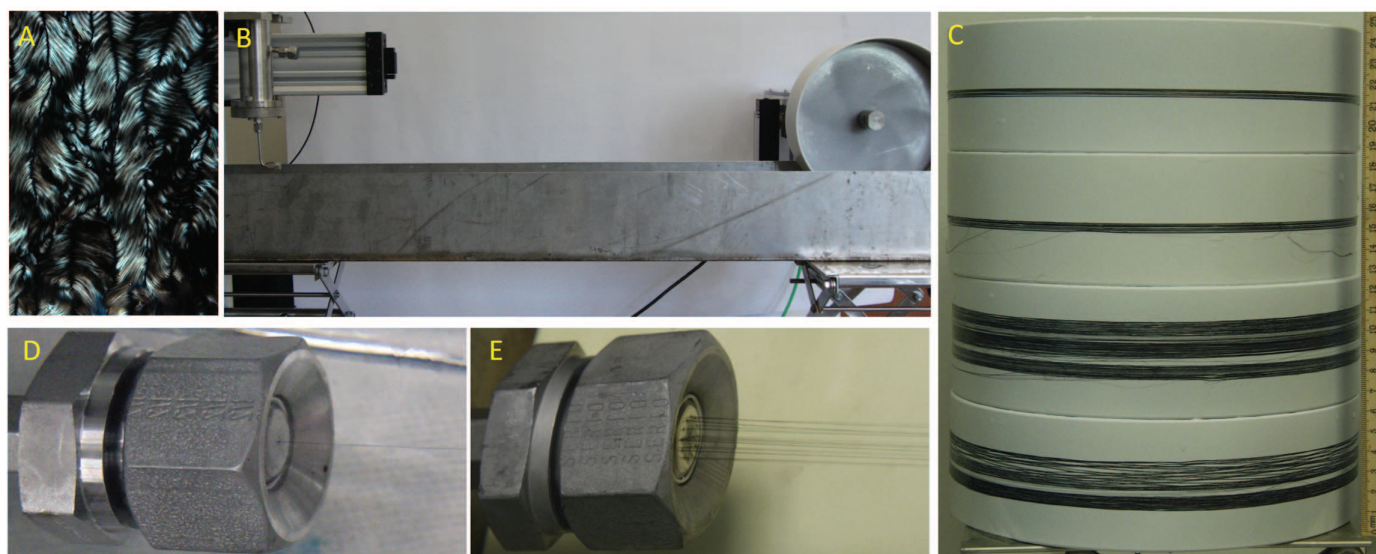


Fig. 1. (A) Light micrograph of a birefringent fiber spinning dope (3 wt % CNT in chlorosulfonic acid). (B) Fiber spinning set-up. The fluid is extruded from the spinning chamber (left) through a spinneret immersed in a coagula-

tion bath; the fiber is continuously collected on the winding drum (right). (C) Winding drums with collected fibers (100 to 500 m on each drum). (D and E) Close-up view of a single- and 19-filament spinning.

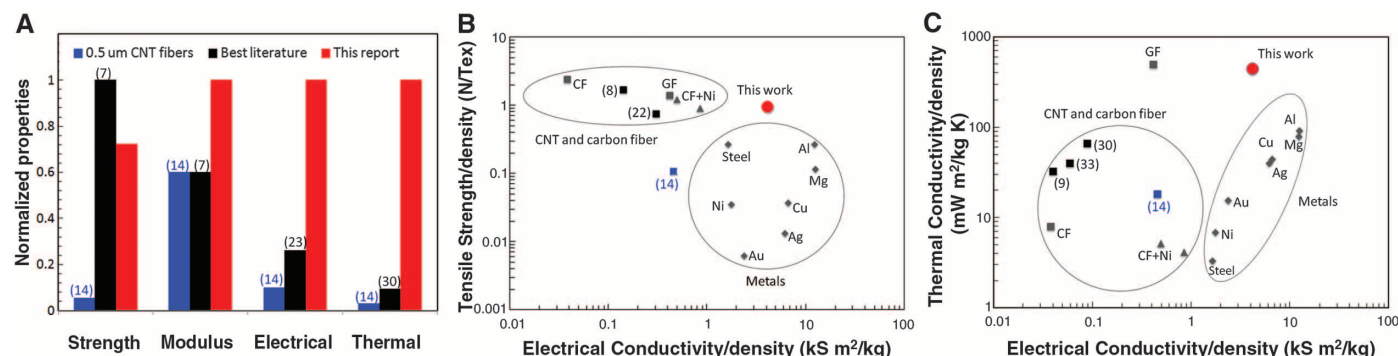


Fig. 2. Properties of continuous, neat CNT fibers. Black denotes literature values (7, 8, 9, 22, 23, 30, 33), blue denotes earlier wet-spun 0.5- μm CNT fibers (14), and red denotes fibers in this report. (A) Comparison of properties normalized to the highest value. (B) Ashby plots of specific tensile strength versus specific electrical conductivity of metals (gray diamonds), pitch (GF) and PAN (CF) carbon

fibers (gray squares), nickel-coated CF (gray triangles), CNT fibers (black and blue squares), and CNT fibers from this report (red circle). Carbon and CNT fibers fall in a high-strength, low-conductivity region; metals define a high-conductivity, low-strength region. (C) Specific electrical conductivity versus specific thermal conductivity Ashby plot, showing distinct regions for metals and carbon fiber.

transmission electron microscopy (TEM)]. HR-SEM revealed that the fiber consists of well-aligned, thin CNT fibrils (Fig. 3, A and B) (typical diameter of 10 to 100 nm and length $>50\ \mu\text{m}$), similar to wet-spun 0.5- μm CNT fibers (14), as well as high-strength polymeric fibers (32). WAXD showed sharp reflections at $2\theta = 25.3^\circ$ (Fig. 3C and fig. S14), due to planes perpendicular to the nanotube axis (15). The azimuthal scan of this peak gave a full width at half maximum (FWHM) of 9.4° (which corresponds to a Herman orientation factor of 0.986), averaged over four different points along a single fiber (lowest and highest values were 7.3° and 11.2°). Electron diffraction on isolated single fibrils (peeled off the fibers) displayed better order (FWHM = 5°), indicating slight misalignment of fibrils within the fiber (Fig. 3C). This compares to FWHM = 31° for wet-spun 0.5- μm CNT fibers (14) and FWHM between 10° and 14° for solid-state spun fibers (33). We also quantified packing fraction by calculating a maximum theoretical packing density of $1.5\ \text{g/cm}^3$ from high-resolution TEM on the starting CNT material (15). Hence, the fiber density ($1.3\ \text{g/cm}^3$) is $\sim 90\%$ of the theoretical close-packed density. We verified the high packing fraction by visualizing the fiber cross section. The images showed occasional $\sim 100\text{-nm}$ voids, but no larger voids (Fig. 3, D and E). This packing density for neat carbon nanotube fibers compares with 78% for wet-spun 0.5- μm CNT fibers (14) and $\sim 50\%$ for solid-state fibers (3).

To understand the relative effect of CNT alignment, packing, and doping on the conduction mechanism in these fibers, we studied the temperature-dependent conductivity of annealed fibers and isotropic films, as well as acid-doped and iodine-doped fibers (differing in extent of doping). All samples show two regimes (Fig. 4A): At low temperature the conductivity rises with temperature (semiconducting behavior), whereas at high temperature the conductivity drops (metallic behavior). The crossover temperature (indicated by an arrow) is highest in the annealed fiber and film and lowest in the iodine-doped fiber. The low-temperature behavior can be understood in terms of carrier hopping or tunneling, i.e., inter-CNT transport, which can be substantially affected by mismatch of CNT types (metallic versus semiconducting) and degree of alignment. The high-temperature, metallic behavior reflects diffusive, intra-CNT transport, for which the conductivity is limited by electron-phonon scattering. Each temperature-dependent curve can be well fitted (15) by an equation (table S2) that combines the two regimes (34), as shown in Fig. 4A. The annealed fiber is ~ 3.5 times more conductive than the annealed film. This is due to the CNT alignment in the fiber, which increases CNT density and overlap and decreases their spacing, facilitating inter-CNT transport. Doping the fiber by residual acid or iodine further improves the conductivity by $\sim 5\text{-}$ to 10-fold because doping increases the intra-CNT conductivity of the semi-

conducting CNTs and also increases disorder that helps relax the momentum conservation required for inter-CNT transport (35). These advantages of doping seem to prevail over the conductivity loss due to enhanced electron-impurity scattering.

Above $\sim 1\ \text{K}$, the thermal conductivity is dominated by phonon transport and hence not closely correlated to the electrical conductivity (Fig. 4B). We observe in a macroscopic sample a crossover (at 250 to 300 K) from impurity-phonon scattering to three-phonon Umklapp scattering, similar to the behavior of a single multiwalled carbon nanotube (MWNT) (36). The conductivity is about 20% of the single-MWNT value, possibly due to quenching by the inter-CNT coupling, but 10 to 100 times that of other macroscopic CNT samples, usually limited by weak inter-CNT transport due to misalignment. Acid removal by annealing roughly doubles the fiber thermal conductivity, presumably due to reduced phonon-impurity scattering; surprisingly, annealing in the presence of iodine yields comparable improvement (15), showing that both high electrical and thermal conductivity can be attained on the same fiber.

We demonstrate the multifunctional properties of our fibers by supporting and wiring a light-emitting diode (LED, 46 g) and by fabricating cold electron-emitting cathodes. The LED was supported by two CNT fibers [$(24 \pm 1)\text{-}\mu\text{m}$ diameter, Fig. 4C]. The stress in each fiber was

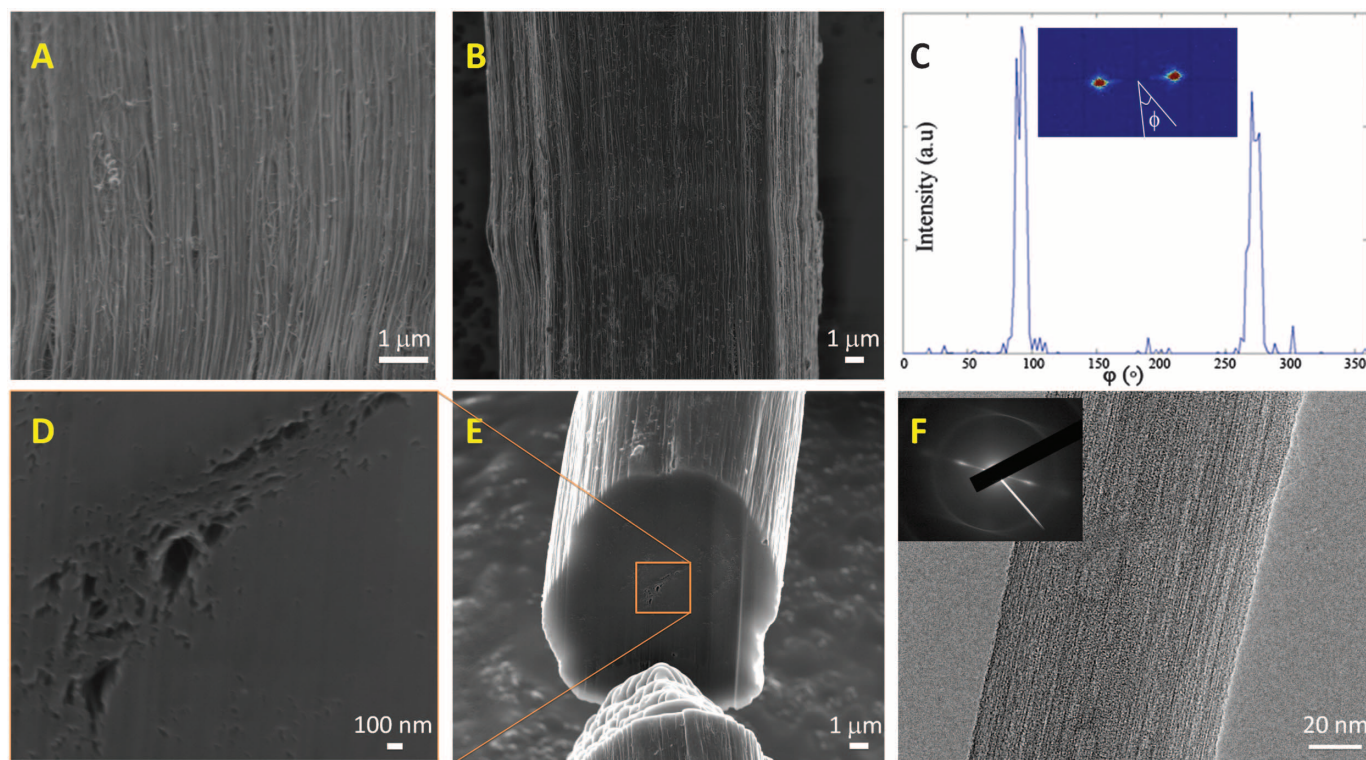


Fig. 3. (A) High- and (B) low-magnification SEM showing the typical morphology of CNT fibers composed of $\sim 100\text{-nm}$ -thick fibrils aligned along the fiber axis. (C) Single-fiber x-ray diffraction (inset) and azimuthal scan showing the high fiber alignment. (D and E) SEM images showing fiber's cross

section after cutting by focused ion beam. There are no micrometer-sized voids and few hundred-nanometer-sized voids. (F) Single-fibril TEM micrograph and electron diffraction (inset) showing near-crystalline packing within a fibril.

~500 MPa, well above the breaking strength of copper wires, but the filament resistance was low enough to run 30-mA current (6.6×10^3 A/cm² current density) and light the device.

A field-emitting device (15) was fabricated in a cathode (fiber)–anode configuration (Fig. 4D). The emitted current density was 5.8×10^3 A/cm² (3.6 mA from a 9-μm-diameter fiber) at 0.86 V/μm and 1-mm anode-cathode distance (Fig. 4E). This current density is more than two orders of magnitude higher than that of wet-spun 0.5 μm CNT fibers (14), graphite fibers (a common material for large area cathodes), and typical solid-state spun CNT fibers (37–41). The high current density of our fibers is due to the high electrical and thermal conductivity, which reduces the fiber temperature by generating less Joule heating and by dissipating the heat efficiently, preventing tip failure.

High performance is only one of the two requisites for technology adaptation. Ease of manufacturing is key for cost control through process scale-up (15). We demonstrated scalability of solution spinning by using a multihole spinneret under the same conditions as for the single hole

spinning—same linear extrusion rate (~10 m/min) per hole and spinneret diameter (Fig. 1E). The only required change was a simple switch of the spinning plate, with no alteration of any other hardware. The produced 19-filament fibers showed properties comparable to those of the mono-filament ones.

The combined achievement of CNT fiber multifunctionality and a scalable manufacturing process is a major step toward macroscopic CNT-based materials. As with high-performance polymeric fibers, optimal morphology (alignment, packing density, lack of impurities, and high-quality molecular constituents) is crucial for the final CNT fiber properties. Yet, the fiber properties are still a fraction (~2 to 20%) of the ultimate intrinsic properties of individual CNTs and the expected properties of their fibers. Increasing fiber strength will require the production of longer, thin, defect-free CNTs (speculatively, length >50 μm, diameter <3 nm), and preferably single-walled carbon nanotubes (SWNTs). Reaching ultimate conductivity may require the synthesis of all-armchair SWNTs. The consistent synthesis of large quantities (gram amounts or higher)

of defect-free CNTs with such control on length, diameter, and chirality is a key technological barrier that must be overcome to enlarge greatly the design space of CNT fibers and to make high-performance macroscopic materials of CNTs.

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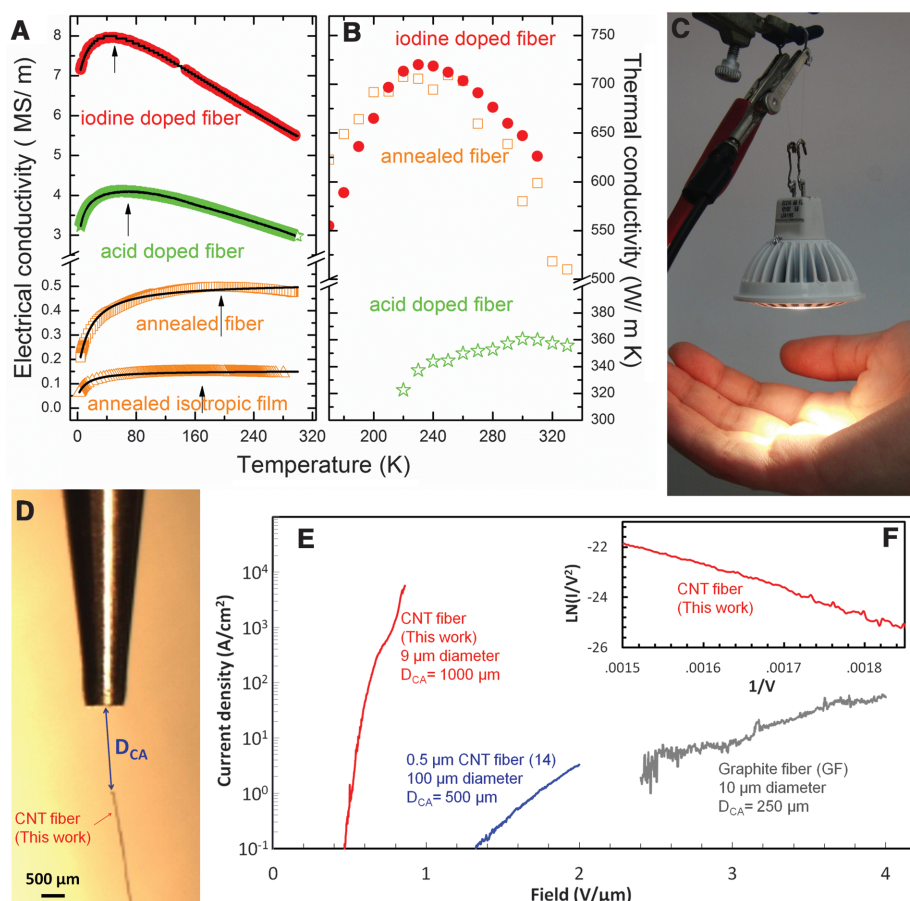


Fig. 4. (A) Temperature dependence of electrical and (B) thermal conductivity of acid-doped, annealed, and iodine-doped fibers and an annealed random film. (C) A 46-g light-emitting diode lit and suspended by two 24-μm-thick CNT fibers. (D) Field emission set-up, with a single-fiber cathode facing the anode. (E) Current density versus field strength of GF (gray), wet-spun 0.5-μm CNT fiber (blue) and our CNT fiber (red). (F) The I - V data in simplified Fowler-Nordheim coordinates fall on a straight line, indicating metallic field emission.

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Supplementary Materials

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Supplementary Text

Figs. S1 to S3
Tables S1 to S3
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Bio-Inspired Polymer Composite Actuator and Generator Driven by Water Gradients

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Here we describe the development of a water-responsive polymer film. Combining both a rigid matrix (polypyrrole) and a dynamic network (polyol-borate), strong and flexible polymer films were developed that can exchange water with the environment to induce film expansion and contraction, resulting in rapid and continuous locomotion. The film actuator can generate contractile stress up to 27 megapascals, lift objects 380 times heavier than itself, and transport cargo 10 times heavier than itself. We have assembled a generator by associating this actuator with a piezoelectric element. Driven by water gradients, this generator outputs alternating electricity at ~0.3 hertz, with a peak voltage of ~1.0 volt. The electrical energy is stored in capacitors that could power micro- and nanoelectronic devices.

Polymeric materials that reversibly change shape, size, or mechanical properties in response to external stimuli have attracted considerable interest because of their potential applications as actuators for biomedical and me-

chanical purposes (1). Based on their energy sources for actuation, responsive polymeric materials can be divided into three classes: electroactive polymers (2, 3), light- or thermal-responsive elastomers (4–9), and pH- or solvent-responsive

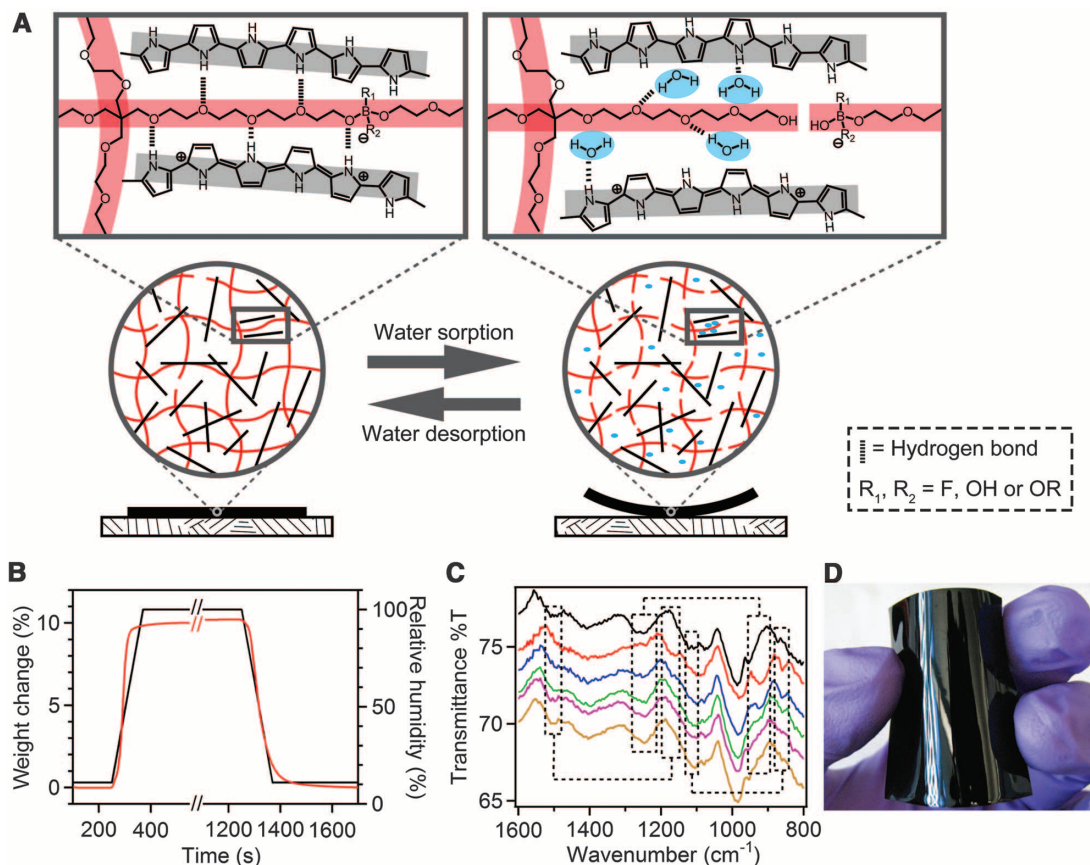
gels (10–14). Many organisms use water-sorption-induced swelling for actuation (15). Several types of water-responsive hydrogels have been developed for actuator fabrication (10), but they exhibit slower response, lower stress generation, and marginal stability than do animal muscle fibers.

Polypyrrole (PPy) is an electroactive polymer with many desirable properties that could allow it to act as an artificial muscle (16, 17). PPy can also absorb water and change its shape, which allows it to drive motion in a rotary actuator (18). However, existing PPy rotary actuators only weakly output mechanical force or power, in contrast to PPy-based electroactuators (16, 18). Inspired by the network structure of animal dermis, in which rigid collagen fibers reinforce an elastic

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Fig. 1. Characterization of PEE-PPy composite films. (A) A PEE-PPy composite film (black) is composed of PPy polymer chains (gray lines) and a PEE-borate network (red lines). The structure changes (involving H bonds and borate ester bonds) in response to water (blue dots) sorption and desorption. (B) PEE-PPy weight change (red) synchronizes with air humidity change (black). (C) ATR-IR spectra showing H/D exchange between the PEE-PPy film and water vapor. Top to bottom: before D₂O exposure and 0, 1, 2, 3, and 4 min after D₂O exposure. Dashed lines indicate the three pairs of shifting peaks. (D) A PEE-PPy film maintains its flexibility and mirrorlike surface after 6 months of open storage.



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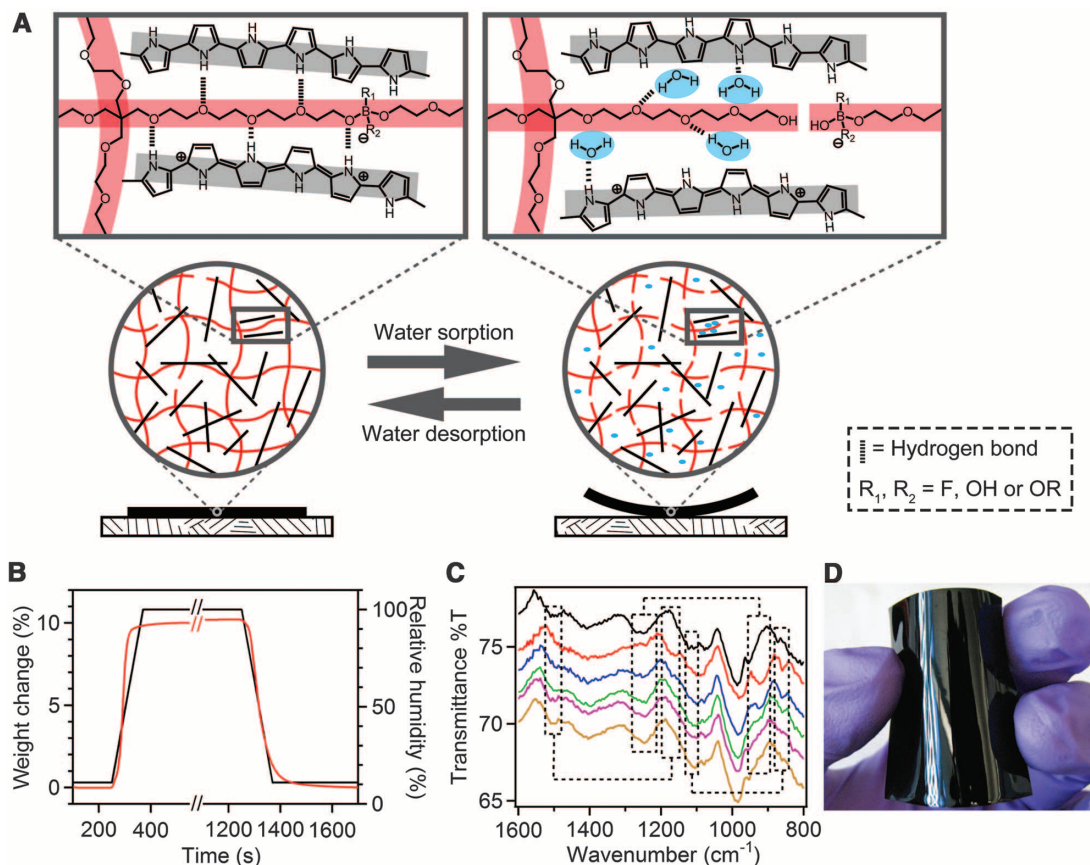
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Fig. 1. Characterization of PEE-PPy composite films. (A) A PEE-PPy composite film (black) is composed of PPy polymer chains (gray lines) and a PEE-borate network (red lines). The structure changes (involving H bonds and borate ester bonds) in response to water (blue dots) sorption and desorption. (B) PEE-PPy weight change (red) synchronizes with air humidity change (black). (C) ATR-IR spectra showing H/D exchange between the PEE-PPy film and water vapor. Top to bottom: before D₂O exposure and 0, 1, 2, 3, and 4 min after D₂O exposure. Dashed lines indicate the three pairs of shifting peaks. (D) A PEE-PPy film maintains its flexibility and mirrorlike surface after 6 months of open storage.



network of elastin microfibrils to form a sturdy and flexible material (19), we hypothesized that a composite of a soft water-responsive gel within a rigid polymer matrix would yield a better water-responsive actuator. We made a dynamic polymer composite of rigid PPy imbedded with a flexible, interpenetrating polyol-borate network (20) that would be responsive to water sorption and desorption (Fig. 1A). The dynamic polyol-borate network formed within the PPy matrix also serves as a macromolecular counterion for PPy. The polyol-borate network is sensitive to water by means of hydrolysis and reforming of the borate ester cross-linking hub upon water sorption and desorption, which changes the mechanical properties of the composite (Fig. 1A). Intermolecular hydrogen bonding between the polyol-borate network and PPy also modulates intermolecular packing of the polymer composite to alter its mechanical properties in response to water. Therefore, the polymer composite exhibits fast, reversible, and dramatic mechanical deformation and recovery in response to environmental moisture, visually reminiscent of “fast twitch” muscle activity.

The free-standing composite polymer film was synthesized by electropolymerization of pyrrole in the presence of a polyol-borate complex. The polyol was pentaerythritol ethoxylate (PEE), a four-armed ethylene glycol oligomer (molecular weight ~ 800), which can coordinate with boron(III) species to form a dynamic polymer network as

indicated by peak shifts in ^{11}B nuclear magnetic resonance (NMR) spectra (20) and increased viscosity (21) (figs. S1 and S2). We hypothesized that the anionic PEE-borate complex could be electrically attracted to the cathode and trapped in the growing PPy matrix as macromolecular counterions. We attempted to reduce the PPy matrix and export small counterions by applying a negative electrical potential to the PEE-PPy film for 1 hour in an electrolytic solution (22). The treated PEE-PPy film showed minimal change in weight, conductivity, and mechanical properties, which suggested that the counterion is a polymeric entity and cannot be electrically transported out of the PPy matrix (22). In contrast, when this reduction-resistant PEE-PPy film was soaked in 90°C water for 1 hour, its weight, conductivity, and mechanical properties significantly decreased (fig. S3 and table S1). PEE and boric acid were identified in the soaked water sample by NMR and infrared (IR) spectroscopy (figs. S4 and S5). The average amount of PEE in the initial PEE-PPy film was $\sim 12\%$ by weight. The leached PEE and boric acid components indicated hydrolysis of the polymeric counteranion, suggestive of the water-responsive nature of the PEE-borate network (Fig. 1A) (20).

Monitored by a quartz crystal microbalance with a humidity module, the PEE-PPy film rapidly absorbed up to 10% of water by weight from humid air. The film's weight change synchro-

nized with the humidity change (Fig. 1B), indicating the film's instant response to water vapor. To probe the polymer's chemical structure change in response to water, a PEE-PPy film was immersed in D_2O for 10 s, then taken out and examined by attenuated total reflectance (ATR)-IR. After D_2O exposure, three IR peaks related to O-H or N-H bending disappeared and three new peaks appeared (fig. S6 and table S2). The peak shifting was due to the hydrogen-deuterium (H-D) exchange of active protons in OH and NH. The isotopic ratio ($v_{\text{H}}/v_{\text{D}}$) of the three peaks was smaller than the theoretical value ($v_{\text{H}}/v_{\text{D}} = 1.37$) for free O-H or N-H bonds, suggesting that these active protons were involved in H bonding (23). When this PEE-PPy film was left in ambient air [relative humidity (RH) $\sim 20\%$], the D peaks quickly shifted back to corresponding H peaks within 4 min (Fig. 1C), without contacting any liquid water. This fast H-D exchange demonstrated that the PEE-PPy film was continuously and rapidly “breathing” water from the air, which should manifest in a fast and reversible response to environmental water concentration changes. The film was also characterized by Raman spectroscopy, conductivity measurements, and mechanical analysis (see the supplementary materials for details), which showed a combination of moderate conductivity (30 S/cm), high tensile strength (115 MPa), and good flexibility (elongation at break = 23%). The film also

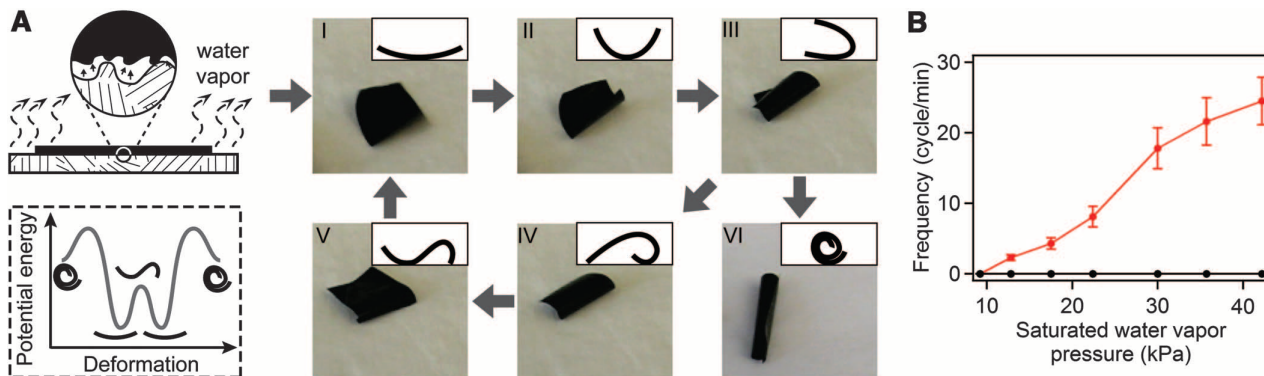


Fig. 2. Locomotion of a PEE-PPy film on a moist substrate. (A) Representative images and sketches of the film's multistage locomotion and a schematic diagram of the film's elastic potential energy. (B) Flipping frequency of PEE-

PPy (red) and PPy (black) films correlated with saturated water vapor pressure at each substrate temperature ($n = 5$). One flipping cycle refers to a motion process starting from stage I through stage V and back to stage I.

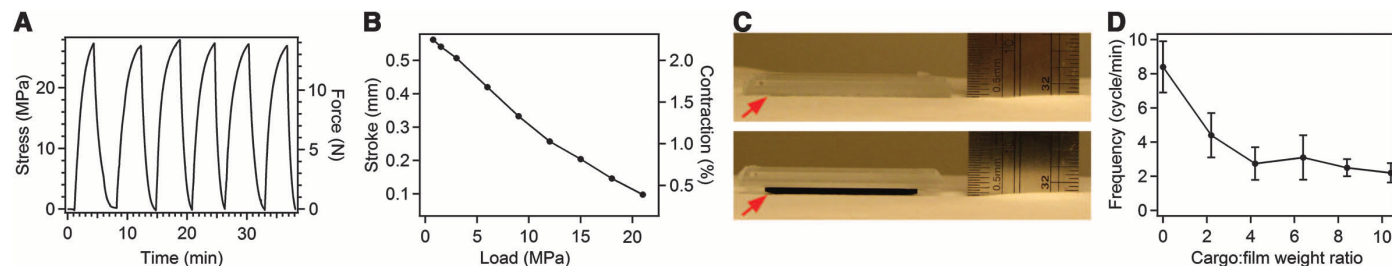


Fig. 3. Mechanical performance of a 25-mg PEE-PPy film actuator. (A) The contractile stress and force generated in the film upon water sorption and desorption. (B) The load-dependent stroke of the actuator contraction. (C) Images of the actuator under microscopy glass slides

(top image) buckling and lifting the slides up for ~ 2 mm (bottom image). The red arrows indicate the position of the 30- μm -thick actuator. (D) The flipping frequency of the actuator with cargo loading ($n = 5$).

showed good stability while exposed to air and ambient humidity for 6 months (Fig. 1D and fig. S7).

The PEE-PPy film spontaneously and continuously flipped and navigated over a moist nonwoven paper substrate (movie S1). Film motility required the water gradient, rather than just water: In a closed chamber saturated by water vapor, the PEE-PPy film folded into a roll and remained static. One locomotive cycle of the film was generally composed of five stages (Fig. 2A, I to V). As a PEE-PPy film contacted the moist substrate, the bottom face absorbed more water than the top face, which caused asymmetric swelling and film curling away from the substrate (I). Thus, the film/substrate contact area decreased and the film's gravity center rose, which led to mechanical instability (II) and eventually caused the buckled film to topple over (III). Asymmetric water sorption was repeated at the film/substrate interface, which cooperated with water release from the raised part of the film to generate horizontal movement (IV). Finally, most of the contact area curled up, and the film fell back to the substrate with a new face down (V) to start a new cycle (I). During the flipping process (stages I to V), both faces of the film were equilibrating with water in the substrate and in the lower-humidity air above. Thus, the water gradient between substrate and air was reflected in the asymmetric film deformation that drove the film locomotion, with the cooperation of film gravity and friction with the substrate. Analysis of the frequency of film flipping motion as a function of the saturated water vapor pressure P_s (proportional to the water evaporation rate, eq. S1) at each substrate temperature indicated a roughly linear correlation (Fig. 2B), suggesting that the film locomotion could be regulated by controlling the water evaporation rate. If the air close to the substrate was water-saturated, the PEE-PPy film would fold into a roll (VI), which possessed high elastic energy of 21 to 76 J/kg and could partially release the energy in a sudden and quick leap (Fig. 2A energy diagram, movie S2, and eqs. S2 and S3).

Water gradients were most effective in driving PEE-PPy film locomotion. Volatile polar organic solvents (such as alcohols, ketones, and esters) also caused the PEE-PPy film to swell and buckle, but its translational motion was significantly weaker or unable to be completed. This can be explained by the interpenetrating polymer structure (the PEE-borate network and its H-bond interaction with the polypyrrole matrix) being more sensitive to water than to other organic solvents (20). We also found that PPy films without an interpenetrating PEE-borate network (18) exhibited marginal film buckling on a moist substrate, with no translational motion, in contrast to a PEE-PPy film that navigated on the same substrate. The different behaviors could be attributed to the observation that water sorption had a bigger softening effect on a PEE-PPy film than on a PPy film (fig. S8). Although dry PPy and PEE-PPy films had similar tensile moduli of ~ 2.3 GPa, the moist PEE-PPy was much softer than the

moist PPy (Young's modulus = 0.65 GPa versus 1.65 GPa, table S1). This difference could also be explained by the interpenetrating polymer structure being broken or weakened upon water sorption and recovered upon water desorption (Fig. 1A). The cooperative switching of the PEE-PPy film between the two states with different physical properties (swelling/soft versus shrinking/stiff) probably contributed to its rapid locomotion. In addition, we also found that a larger PEE-PPy film (9 cm by 9 cm, fig. S9) performed fast locomotion at a similar frequency as a smaller one (2 cm by 4 cm) (movie S3), suggesting that this water-gradient-driven actuator is potentially scalable.

The contractile force and stress generated in a PEE-PPy film actuator were measured on a mechanical analyzer. A 25-mg PEE-PPy film covered by a moist paper was clamped and preloaded with a force of 0.05 N to keep the film tight and straight. When the film was uncovered, a contractile force of up to 14 N was generated by the film's shrinking and stiffening caused by water desorption (Fig. 3A). The maximum stress was 27 MPa, which was about 80 times higher than that of mammalian skeletal muscle (~ 0.35 MPa) (17) and comparable to the maximum stress electrochemically generated in other PPy-based actuators (22 to 34 MPa) (24, 25). When the film was again covered by a moist paper, the contractile force and stress decreased to zero as a result of the water-induced film swelling and softening. The expansion/contraction cycle could be repeated hundreds of times. One full expansion/contraction cycle needed ~ 5 min at room temperature and 20% RH, possibly due to slow water diffusion in the film (18). This does not conflict with the film's fast locomotion, because the locomotion

only needs activation forces in the millinewton level, which can be generated within 0.1 s in the film upon water sorption or desorption. The load-dependent stroke was measured by preloading the moist actuator up to 21 MPa. Upon water desorption, the actuator contracted under a constant load, and the linear relation between stroke and load (Fig. 3B) indicated that the actuator worked in its elastic range. The maximum work during contraction was ~ 73 J/kg, achieved at 9 to 15 MPa loading, which matched the maximum elastic potential energy (76 J/kg) stored in a film roll. These contractions finished in ~ 80 s (fig. S10), which provided an average power density of ~ 0.9 W/kg. Upon water sorption, this 25-mg free film could deform and lift a 9.5 g load to a height of 2 mm within 3 s (Fig. 3C). The work output was 7.6 J/kg and the power density was 2.5 W/kg. In addition, we found that a load of silver wires weighing up to 260 mg attached to the film perimeter was efficiently transported along a substrate-derived water gradient (Fig. 3D, movie S4, and fig. S11).

To probe the capability of the PEE-PPy actuator, we did a theoretical thermodynamic analysis of its water-induced expansion/contraction cycle and came up with the following two equations (see the supplementary materials for detailed analysis)

$$\frac{E \times d}{R} < \frac{(-\Delta G_{\text{cycle}} \times \rho)}{M} \quad (1)$$

$$\frac{E \times d^3}{2R^2} > f_{\text{ad}} \quad (2)$$

E , d , and R are the elastic modulus, thickness, and curvature of the buckled actuator, re-

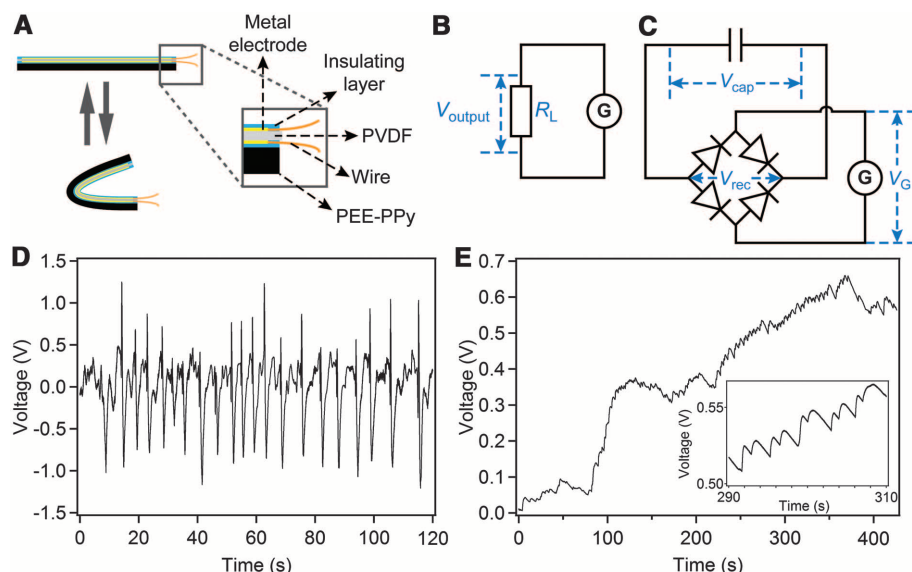


Fig. 4. Design and performance of a water-gradient-driven generator. (A) The assembly of a piezoelectric PVDF element with a PEE-PPy actuator to form the generator. (B) The connection of the generator with a 10-megohm resistor as load. (C) The configuration of the rectifying circuit and charge storage capacitor. (D) The generator's output voltage onto the 10-megohm resistor. (E) Voltage across a capacitor when being charged by the generator. The inset shows a stepwise increase in the capacitor voltage accompanying each cycle of the energy conversion process.

spectively. ρ and M are the density and molecular weight of water, respectively. ΔG_{cycle} is the molar Gibbs free-energy change of absorbed water during one expansion/contraction cycle. f_{ad} is the adhesive force coefficient between PEE-PPy films and moist substrates. Given a certain E , R , and f_{ad} , Eqs. 1 and 2 roughly define a theoretical maximum and minimum limit on the required thickness of the actuator to perform fast locomotion. In practice, we found that the optimal thickness for PEE-PPy actuators on a moist paper was roughly 15 to 40 μm . Actuators thinner than 15 μm tended to stick to the moist paper, whereas actuators thicker than 40 μm showed significantly slower locomotion.

Because this PEE-PPy actuator could continuously extract chemical potential energy out of ambient water gradients to perform mechanical work, it should be able to drive a piezoelectric element to convert the mechanical energy into electrical energy. A 9- μm -thick piezoelectric polyvinylidene difluoride (PVDF) film was metallized, wired, and insulated on both faces (Fig. 4A). A 27- μm -thick PEE-PPy actuator was attached to one face of the PVDF element. When placed on a moist substrate with the actuator facing down, the actuator bent and stretched the PVDF element repeatedly (movie S5), generating an open-circuit voltage up to 3 V. A 10-megohm resistor was loaded onto this generator (Fig. 4B), and the peak output reached ~ 1.0 V (Fig. 4D). Analysis indicated that the frequency of the alternating voltage signal was ~ 0.3 Hz (fig. S12), which matched the motion frequency of the generator (movie S5). The average power output was 5.6 nW (fig. S13), which corresponded to a power density of 56 $\mu\text{W/kg}$ for the 100-mg generator. In contrast, the same PVDF element did not move on the moist substrate, and the recording showed only noise (fig. S14), with analysis of this background noise giving an average power output of 0.015 nW (fig. S15). The

generated alternating electrical pulses by the generator were rectified using a commercial full-wave bridge rectifier, then stored in a 2.2 μF capacitor (Fig. 4C). Within 7 min of charging, the voltage of the capacitor was saturated to ~ 0.66 V (Fig. 4E). This was lower than the peak output voltage of the generator, possibly due to voltage drop across the rectifying diodes and/or current leakage of the capacitor.

This PEE-PPy polymer composite system features an interpenetrating network of a rigid polymer with a soft, hydrolytically sensitive polymer that can perform water-gradient-induced displacement, converting the chemical potential energy in water gradients to mechanical work. Besides mechanical vibration energy, the generator based on this powerful actuator can use ubiquitous low-temperature water gradients as its energy source, in contrast to state-of-the-art piezoelectric energy scavengers that rely solely on mechanical vibration energy (26). Thus, the water-gradient-driven actuator and generator demonstrated potential applications as sensors, switches, and power sources for ultralow-power devices.

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Supplementary Materials

www.sciencemag.org/cgi/content/full/339/6116/186/DC1
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Supplementary Text
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Tables S1 and S2
Eqs. S1 to S3
Movies S1 to S5
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Sequence-Specific Peptide Synthesis by an Artificial Small-Molecule Machine

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The ribosome builds proteins by joining together amino acids in an order determined by messenger RNA. Here, we report on the design, synthesis, and operation of an artificial small-molecule machine that travels along a molecular strand, picking up amino acids that block its path, to synthesize a peptide in a sequence-specific manner. The chemical structure is based on a rotaxane, a molecular ring threaded onto a molecular axle. The ring carries a thiolate group that iteratively removes amino acids in order from the strand and transfers them to a peptide-elongation site through native chemical ligation. The synthesis is demonstrated with $\sim 10^{18}$ molecular machines acting in parallel; this process generates milligram quantities of a peptide with a single sequence confirmed by tandem mass spectrometry.

Cells achieve the sequence-specific synthesis of information-rich oligomers and polymers through the operation of complex

molecular machines that transcribe information from the genetic code (1). The most extraordinary of these is the ribosome (2–4), a ~ 2.6 -MD

(bacterial) to ~ 4.3 -MD (eukaryotic) molecular machine found in all living cells that assembles amino acids from tRNA building blocks into a peptide chain with an order defined by the sequence of the mRNA strand that it moves along. Artificial small-molecule machines (5) have previously been used to store information (6, 7) and do mechanical work (8–11); others have been employed in synthesis to processively epoxidize an unsaturated polymer (12, 13), switch “on” and “off” catalytic activity (14–17), and change the handedness of a reaction product (18). Large synthetic DNA molecules have been used to guide the formation of bonds between unnatural building blocks (19–22) and assemble

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gold nanoparticles in particular sequences (23). Here, we report on the design, synthesis, and operation of a rotaxane-based small-molecule machine in which a functionalized macrocycle operates on a thread containing building blocks in a predetermined order to achieve sequence-specific peptide synthesis. The design of the ar-

tificial molecular machine is based on several elements that have analogs in either ribosomal (2–4) or nonribosomal (24) protein synthesis: Reactive building blocks (the role played by tRNA-bound amino acids) are delivered in a sequence determined by a molecular strand (the role played by mRNA). A macrocycle ensures pro-

cessivity during the machine's operation (reminiscent of the way that subunits of the ribosome clamp the mRNA strand) and bears a catalyst—a tethered thiol group—that detaches the amino acid building blocks from the strand and passes them on to another site at which the resulting peptide oligomer is elongated in a single specific

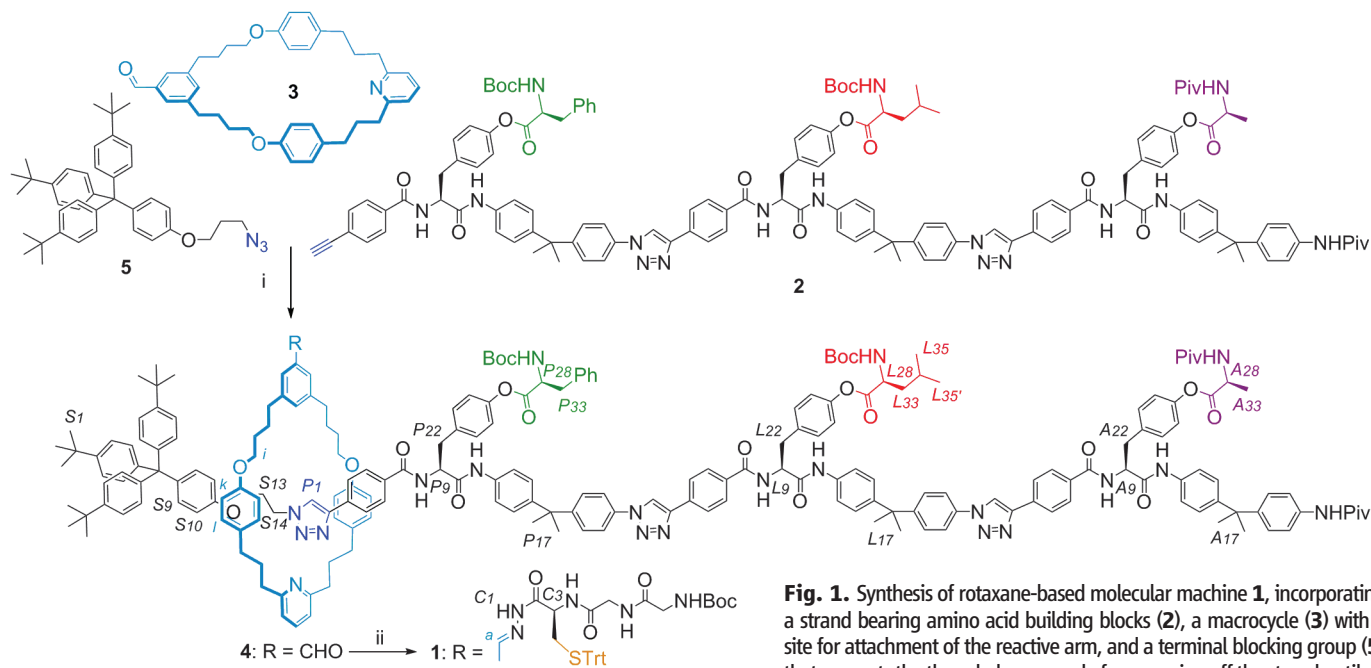
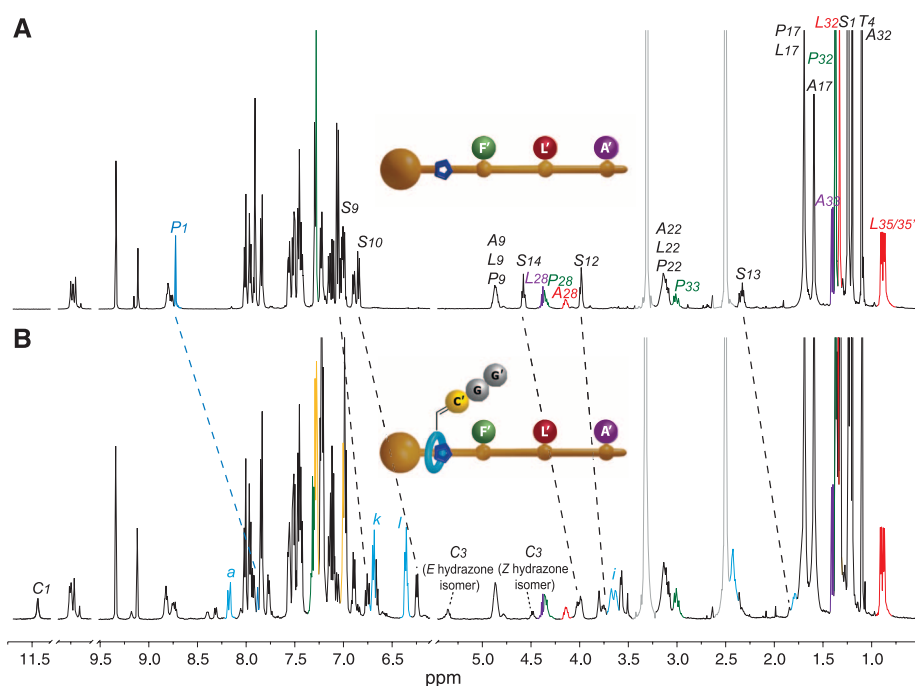


Fig. 1. Synthesis of rotaxane-based molecular machine **1**, incorporating a strand bearing amino acid building blocks (**2**), a macrocycle (**3**) with a site for attachment of the reactive arm, and a terminal blocking group (**5**) that prevents the threaded macrocycle from coming off the strand until all of the amino acid groups have been cleaved. Structure **1** is shown as the major diastereomer; a small amount of epimerization (<5%) of some of the acyl amino units occurs during incorporation into the track. Boc, $\text{CO}_2\text{C}(\text{CH}_3)_3$; Piv, $\text{COC}(\text{CH}_3)_3$; Trt, CPh₃; Ph, phenyl. Reaction conditions: (i) $\text{Cu}(\text{CH}_3\text{CN})_4\text{PF}_6$ in dichloromethane:*t*-butanol (2:1), room temperature, 4 days, 30%. (ii) PhNH₂ (catalyst), BocGlyGlyCys(S-Trt)NHN=CHC₆H₄OCH₃ in 3:1 dimethylsulfoxide: aqueous 2-(*N*-morpholino)ethanesulfonic acid buffer (pH 6.0), 60°C, 2 days, 90%. The italicized letters indicate key signals in the ¹H NMR spectrum shown in Fig. 2B. For the full lettering scheme and assignments, see the supplementary materials, page S28 (26).

Fig. 2. Proton NMR spectrum of (A) the noninterlocked thread and (B) rotaxane **1**, in *d*₆-dimethylsulfoxide (500 MHz, 298 K). Rotaxane **1** exists in both *E*- and *Z*-hydrazone forms. The assignments correspond to the lettering shown in Fig. 1. C', *S*-Trt-cysteine; G', *N*-Boc-glycine; F', *N*-Boc-phenylalanine; L', *N*-Boc-leucine; A', *N*-Piv-alanine. ppm, parts per million.



sequence, through chemistry related to non-ribosomal peptide synthesis (24).

The chemical structure of the artificial molecular machine, **1**, is shown in Fig. 1. Strand **2** bears three amino acids attached to the track by weak phenolic ester linkages (25) and separated from each other by rigid spacers that minimize the possibility of the reactive arm of the machine coming into contact and reacting with a building block out of sequence (26). Macrocycle **3** contains an endotopic pyridine group that directs the

threading of strand **2** during the Cu(I)-catalyzed cycloaddition of the terminal alkyne with the azide-bearing stopper group **5**, leading to the assembly of rotaxane **4** in 30% yield (Fig. 1, i). This active template (27, 28) strategy ensured that the resulting threaded structure did not have residual attractive intercomponent interactions that would tend to localize the position of the ring rather than allow it to move freely up and down the strand between blocking groups. Once we assembled the macrocycle-strand-stopper

conjugate **4**, we used reversible hydrazone exchange to introduce a cysteine derivative bearing the reactive arm [a trityl (Trt)-protected thiol group] and the site for peptide elongation [a *tert*-butoxycarbonyl carbamate (Boc)-protected amine at the end of a glycylglycine residue] (Fig. 1, ii). The fully assembled machine **1** is stable in its protected form, with upfield shifts of the H_{P1} triazole and nearby $H_{S12-S14}$ proton signals evident in the 1H nuclear magnetic resonance (NMR) spectrum on account of shielding from the phenyl rings of the macrocycle, confirming that the ring is trapped in the region of the strand between the terminal stopper and the Boc-phenylalanine ester (Fig. 2).

We used acid-catalyzed cleavage of the Boc and trityl protecting groups (Fig. 3, i) to activate the molecular machine and then allowed it to operate (Fig. 3, ii) at 60°C under microwave heating in a 3:1 acetonitrile:dimethylformamide solution in the presence of *N,N*-diisopropylethylamine (a non-nucleophilic base) and *tris*(2-carboxyethyl)phosphine (a reducing agent that cleaves any disulfide bonds formed through thiol oxidation). The design of the machine is such that once the thiolate residue of the cysteine group (**6a**) is deprotected, it is poised to undergo a transacylation reaction with the first amino acid phenolic ester that blocks the macrocycle's path on the track (**6b**). We hypothesized that the subsequently formed phenylalanine thioester (**6c**) would be able to react further, transferring the amino acid by native chemical ligation (29) to the glycylglycine amine group by an 11-membered-ring transition state [the dipeptide spacer between the cysteine residue and the amine of the peptide-elongation site was introduced because native chemical ligation is reported to be very slow via 8-membered-ring transition states (30)]. This sequence simultaneously transfers the amino acid to the end of the growing peptide (**6d**) and regenerates the catalytic thiolate group, ready for the cleavage and transfer of further building blocks. *S-N* acyl transfer is a key feature of nonribosomal peptide synthesis (24).

Once the covalent bond connecting an amino acid to the strand is broken, the macrocycle is able to move further along the track until its path is blocked by the next amino acid group (**6d**). The *O-S* acyl transfer/*S-N* acyl transfer/catalyst regeneration/ring movement process continues (**6e** to **6h**) until the last amino acid on the track is cleaved (**6h**) and the macrocycle detaches from the strand (**8**) with the newly formed, full length, peptide attached (**7**). The artificial molecular machine synthesizes the peptide from the C terminus to the N terminus, the opposite direction of ribosomal translation (2–4).

After a 36-hour operation of **6a** at 60°C, no starting material remained, as shown by high-performance liquid chromatography (HPLC), and two major products were isolated from the reaction mixture (26). We used 1H NMR spectroscopy and mass spectrometry to identify one product as the completely deacylated thread, **8**. The other product had a 1H NMR spectrum and molecular

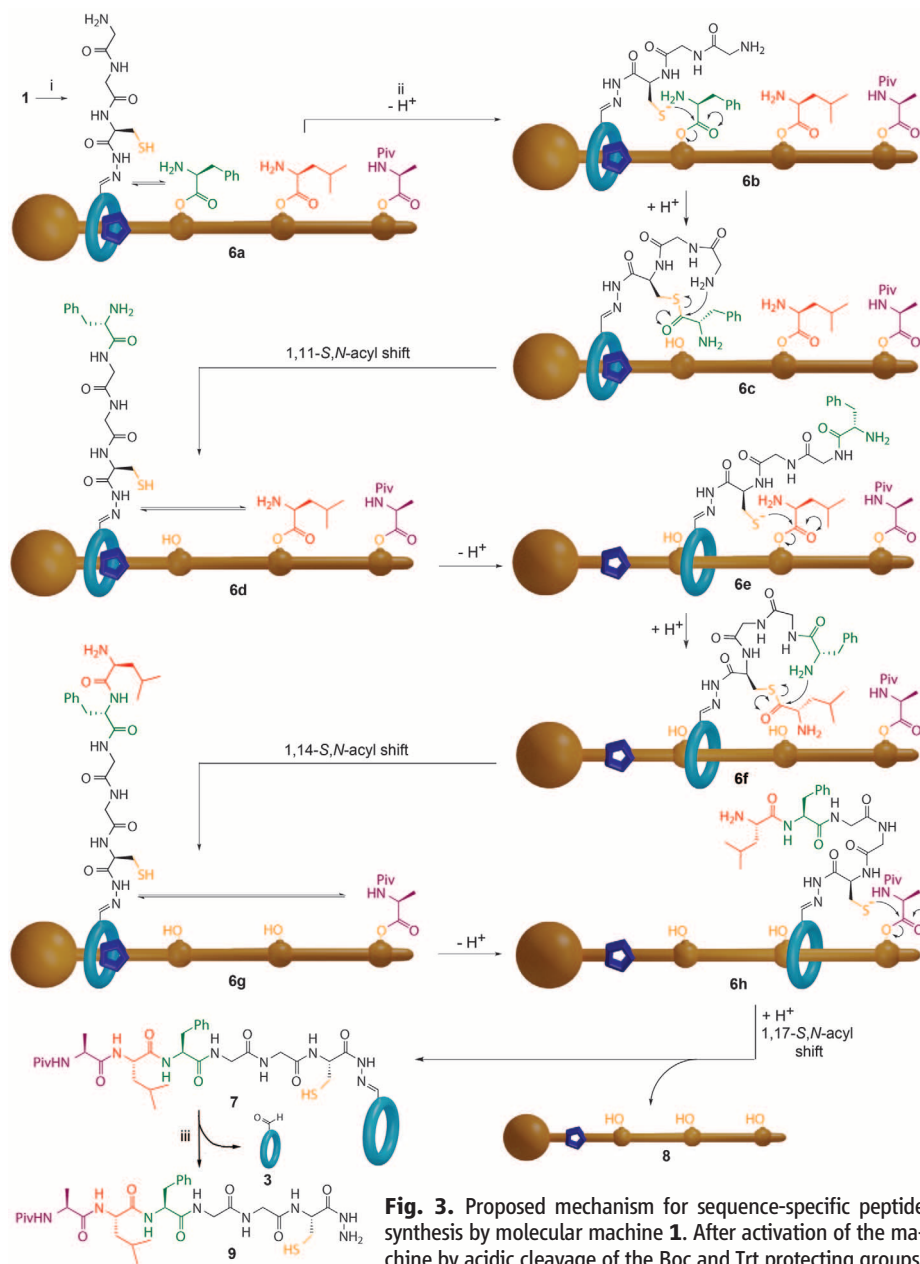


Fig. 3. Proposed mechanism for sequence-specific peptide synthesis by molecular machine **1**. After activation of the machine by acidic cleavage of the Boc and Trt protecting groups, under basic conditions successive native chemical ligation reactions transfer the amino acid building blocks to the peptide-elongation site on the macrocycle in the order they appear on the strand. Once the final amino acid is cleaved, the macrocycle bearing the synthesized oligopeptide **7** dethreads from the strand. The hydrazide peptide **9** is subsequently released from the macrocycle by hydrolysis. Reaction conditions: (i) 20% CF_3CO_2H in dichloromethane, room temperature, 2 hours, 100%. (ii) $((CH_3)_2CH)_2NEt$, $(HO_2CCH_2CH_2)_3P$ in 3:1 acetonitrile:dimethylformamide, 60°C, 36 hours. Et, ethyl. (iii) 30% CF_3CO_2H in 3:1 dichloromethane:water, room temperature, 18 hours.

After a 36-hour operation of **6a** at 60°C, no starting material remained, as shown by high-performance liquid chromatography (HPLC), and two major products were isolated from the reaction mixture (26). We used 1H NMR spectroscopy and mass spectrometry to identify one product as the completely deacylated thread, **8**. The other product had a 1H NMR spectrum and molecular

weight (Fig. 4) consistent with the macrocycle bearing the hydrazone linked to the hexapeptide (Piv)AlaLeuPheGlyGlyCys, **7** [Piv, COC(CH₃)₃].

To confirm that the product of the molecular machine's operation had the amino acids assembled in the correct order, we used tandem mass spectrometry (MS/MS) to determine the peptide sequence. We then validated the sequence by comparing it with an authentic sample and an isomer in which the order of the Phe and Leu residues was reversed, each prepared unambiguously by conventional peptide synthesis (26). Figure 4A shows the ¹H NMR spectrum of the molecular machine product **7**, and Fig. 4B shows the MS/MS spectrum of one isotope of a 2+ ion of **7** derivatized through the cysteine as an *S,N*-acetal, a species that gave a sufficient signal-to-noise ratio for the MS/MS experiment. Figure 4C shows the superimposition of the MS/MS spectra of a similar isotope and ion from the authentic samples of the macrocycle bearing the sequence (Piv)AlaPheLeuGlyGlyCys (red peaks) and (Piv)AlaLeuPheGlyGlyCys (blue peaks). The difference in the fragmentation masses of the two sequence isomers is

apparent in Fig. 4C (725.73 for the LeuGlyGlyCys-macrocycle, 742.92 for the PheGlyGlyCys-macrocycle), and product **7** was confirmed as corresponding to the intended sequence isomer.

With the use of HPLC-MS analysis of the reaction mixture from the operation of **1**, we did not detect any products corresponding to other peptide compositions (neither different sequences nor peptides with more or less than one Phe, Leu, or Ala residue), indicating that the peptide synthesis occurs overwhelmingly within the confines of the molecular machine. In contrast, a control reaction carried out under identical conditions but using the nonthreaded strand and macrocycle yielded several products, including strands with one or more amino acid groups cleaved, but there was no evidence for the formation of **7** under these conditions. Thus, the threaded architecture of the molecular machine—encompassing the catalytic site, elongation site, and the building block strand—is essential for the sequential peptide synthesis, and the mode of operation of the molecular machine is consistent with the mechanism shown in Fig. 3. The peptide (**9**), still bearing the GlyGlyCys unit at the C terminus, could

subsequently be cleaved from the macrocycle by hydrolysis (Fig. 3, iii).

On a scale of tens of milligrams, we performed the synthesis of the small peptide through autonomous multistep production by artificial small-molecule machine **1**, corresponding to parallel synthesis by ~10¹⁸ machines. Once operation is initiated, the synthetic tasks performed by **1** proceed automatically, requiring no further intervention. As the catalytic thiolate is constrained by the threaded architecture of the machine from reacting with building blocks out of sequence, the act of balancing the rate of reactions with the speed that templates rearrange (19–22) is unnecessary for a rotaxane-based machine.

Rotaxane **1** is a (very) primitive analog of the ribosome. Limitations of the first-generation artificial system include slow kinetics (**1** takes ~12 hours to make each amide bond, compared to the 15 to 20 amide bonds synthesized per second by a ribosome) and loss of the sequence information on the strand as it is translated into the product. Furthermore, the size of oligopeptide that can be produced may ultimately be restricted by the size of the cyclic transition states involved

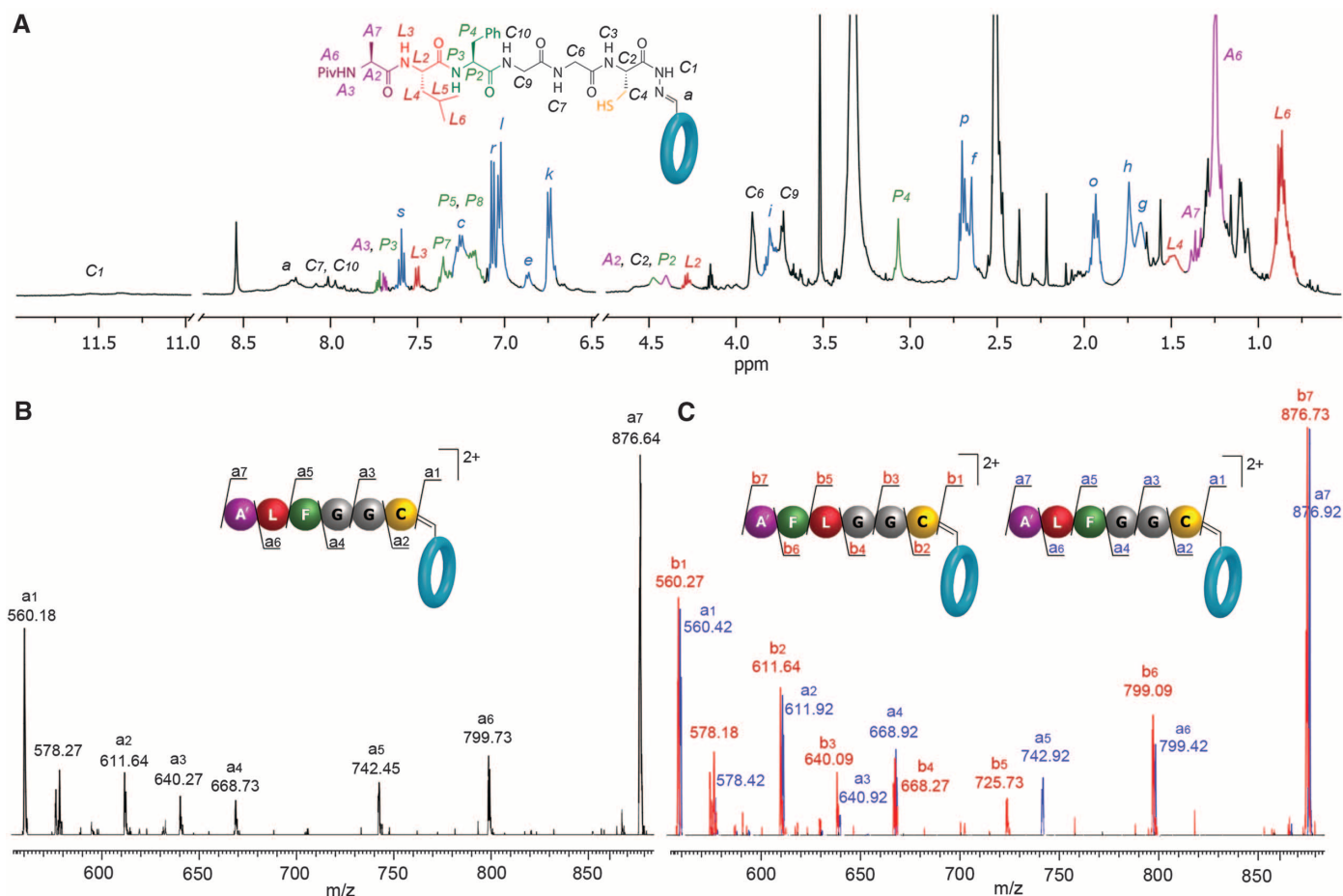


Fig. 4. (A) Proton NMR spectrum of molecular machine operation product **7** in *d*₆-dimethylsulfoxide (500 MHz, 298 K). (B) Tandem mass spectrum of a single isotope of the 2+ ion [mass/charge ratio (*m/z*) = 876.64] of *S,N*-acetal-derivatized **7**. (C) Superimposed tandem mass spectra of 2+ ions of *S,N*-acetal-

derivatized macrocycles bearing the peptide sequences (Piv)AlaPheLeuGlyGlyCys (red; 2+ ion isotope-selected *m/z* = 876.73) and (Piv)AlaLeuPheGlyGlyCys (blue; 2+ ion isotope-selected *m/z* = 876.92), each prepared unambiguously by conventional peptide synthesis.

in S-to-N acyl transfer [although peptide ligation has been successfully used with up to 29-membered cyclic transition states (31)]. Nevertheless, **1** demonstrates that relatively small, highly modular, artificial molecular machines can be designed to autonomously perform iterative tasks in synthesis. The principles employed in the design and operation of **1** should be broadly applicable to other types of monomer and chemical reactions (32).

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Shape-Memory Nanopores Induced in Coordination Frameworks by Crystal Downsizing

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Flexible porous coordination polymers change their structure in response to molecular incorporation but recover their original configuration after the guest has been removed. We demonstrated that the crystal downsizing of twofold interpenetrated frameworks of $[\text{Cu}_2(\text{dicarboxylate})_2(\text{amine})]_n$ regulates the structural flexibility and induces a shape-memory effect in the coordination frameworks. In addition to the two structures that contribute to the sorption process (that is, a nonporous closed phase and a guest-included open phase), we isolated an unusual, metastable open dried phase when downsizing the crystals to the mesoscale, and the closed phase was recovered by thermal treatment. Crystal downsizing suppressed the structural mobility and stabilized the open dried phase. The successful isolation of two interconvertible empty phases, the closed phase and the open dried phase, provided switchable sorption properties with or without gate-opening behavior.

Shape-memory materials alter their morphological appearance in response to an external stimulus (for example, mechanical stress created by macroscopic structural deformation), hold their new temporary shape after the stimulus has been removed, and return to their original morphology in the presence of another external stimulus (1, 2). For instance, a metal alloy can exhibit shape-memory effect if it has two phases that can interconvert reversibly; that is, without requiring atoms to diffuse through the structure. Here, we describe a molecular-scale shape-memory effect (MSME) in nanoporous

framework materials in which the application of an adsorption stress deforms the original shape of the nanopore into a temporary shape, which is maintained even after desorption, and thermal treatment then recovers the original shape.

Our design takes advantage of the flexibility of crystalline porous coordination polymers (PCPs) (3–8), which are assembled from organic spokes and inorganic joints. These flexible PCPs cooperatively reconfigure their framework structures in response to the incorporation of molecules into the nanopores; this adsorption process triggers the deformation of the pore shape. Most flexible

PCPs recover the original structure after the removal of the adsorption stress (that is, the desorption of the guest molecules), which leads to the so-called framework elasticity property (9). The MSME requires that any structural transformation during desorption should be suppressed. We show that crystal downsizing influences the structural mobility, because a reduction in the number of repeating units should be sufficient to regulate the cooperative nature of the structural transformation and the effect of stress.

We fabricated MSME nanopores by crystal downsizing, which regulated the flexibility of the framework, and demonstrated the switchable sorption events based on the presence of two interconvertible pore shapes (Fig. 1). Among the variety of flexible PCPs, we chose a PCP with a twofold interpenetrated framework (10–12)—namely, $[\text{Cu}_2(\text{bdc})_2(\text{bpy})]_n$ (**1**, bdc = 1,4-benzenedicarboxylate, bpy = 4,4'-bipyridine) (13)—that exhibits a cooperative structural transformation from the

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nonporous closed phase to the guest-included open phase with a gate-type abrupt sorption behavior (fig. S1) (14).

We confirmed the identities of both structural phases by single-crystal x-ray diffraction (XRD) for **1** and **1**G [G = *N,N'*-dimethylformamide (DMF), ethanol, or methanol] (1) and that all phases crystallized as triclinic P-1. The framework structure is composed of two identical but distinct frameworks based on six connected dicopper paddlewheel nodes interconnected with bdc in the equatorial plane and bpy in the apical positions. In the open structure of **1**GDMF, the two scaffolds are located near one another (3.42 Å) because of the presence of interframework π - π interactions that produce the accessible channels (Fig. 2, A and B; **1**Gmethanol and **1**Gethanol are shown in fig. S2). The removal of guest molecules leads to the closed phase of **1**, in which the framework undergoes a drastic shearing to minimize the amount of void space and dislocates the center of the void while maintaining the overall framework connectivity (Fig. 2, C and D).

Crystal downsizing of PCPs has previously been accomplished by rapid nucleation with the use of microwaves (15) or ultrasound (16) or by inverse microemulsion with surfactants (17). Our approach, coordination modulation (18), allows the coordination equilibrium at the crystal surface to be controlled with an additive with the same coordination moiety as the linker, which provides highly crystalline meso-sized crystals with a defined crystal surface structure. Micrometer-sized polycrystalline powders of **1** (**1-micro**) were synthesized by first forming a two-dimensional (2D) square grid of $[\text{Cu}(\text{bdc})(\text{S})]_n$ (S = solvent) by reacting copper ions with bdc. Insertion of bpy and an exchange reaction with S formed a 3D framework. Meso-sized crystals of **1** (**1-meso**) were made by adding acetic acid in the first step of the reaction as a modulator to a mixture of copper acetate and H_2bdc (H_2bdc = 1,4-benzenedicarboxylic acid).

As the modulator concentration ratio ($r = 10, 20, 30, 40$ and 50 , where $r = [\text{acetic acid}]/[\text{copper acetate}]$) was increased, the average crystal size of **1-meso** increased from ~ 50 by 50 by 20 nm^3 (**1-meso50**; $r = 10$) to ~ 300 by 300 by 30 nm^3 (**1-meso300**; $r = 50$) (Fig. 3, A to F; figs. S3 to S5; and table S1) (19). Electron diffraction patterns indicated that the shortest dimension of the platelike crystals of **1-meso** corresponded to the Cu-N coordination direction of the framework (fig. S6). Whereas all as-synthesized crystals were identified as the guest-included open phase by powder XRD analysis, the subsequent evacuation of those crystals led to the formation of different structures, depending on crystal sizes (Fig. 3G and fig. S7). As observed in a single crystal of **1**, powders of **1-micro** and the largest meso-sized crystal (**1-meso300**) formed the closed phase. In contrast, the smallest crystal of **1-meso50** maintained the open phase, even after the removal of the guest molecules under vacuum (the open dried phase).

In the intermediate crystal size (between **1-meso300** and **1-meso50**), the contribution of the open dried phase increased with a decrease in the crystal size. We used thermogravimetric analysis to verify the lack of guest inclusion in the open dried phase of **1-meso50**; weight loss did not occur before the decomposition temperature (280°C) (figs. S8 to S19). The meso-sized crystals, **1-meso50**, allowed us to isolate the temporary phase as the open dried phase. Thermal treatment recovered the original closed phase from the open dried phase, which was characterized by variable temperature powder XRD measurements (figs. S20 and S21): (i) The open dried phase was converted into the closed phase by heating to 200°C ; (ii) an intermediate structural phase was not observed; and (iii) the closed phase was maintained, even after cooling to room temperature. Thus, the open dried phase was metastable relative to the closed phase. This thermodynamic analysis was also supported by differential scanning calorimetry measurements for the open dried phase, in which we observed a broad exothermic peak for only the first scan but not for the second scan (fig. S22). We performed a solvent-mediated structural transformation to successfully reproduce the open dried phase: The closed phase of **1-meso50** was immersed in methanol, and the guest methanol molecules were then removed under vacuum (fig. S23). Hence, we demonstrated the MSME of **1-meso50**.

The systematic production of size-controlled mesoscopic crystals of **1** allowed us to investigate the correlation between the crystal size and the sorption properties. We obtained the methanol adsorption isotherm of the conventional micrometer crystals of **1-micro** at 30°C and observed the characteristic sharp uptake of molecules at $P/P_0 = 0.10$, or gate-opening pressure (P/P_0 , relative pressure) (Fig. 4A and figs. S24 to S29). Using an environmentally controlled synchrotron XRD system (fig. S30), we determined that the sharp uptake corresponded to a structural transformation from the nonporous closed phase to the porous open phase in response to methanol accommodation (fig. S31). We then performed methanol adsorption measurements on the closed phase of the size-controlled mesoscopic crystals. As the crystal size decreased from **1-micro** to **1-meso50**, the gate-opening pressure shifted to a higher value. Environmentally controlled synchrotron XRD measurements on closed **1-meso50** revealed that the structural transformation occurred in a higher-pressure region and presented a wider pressure range ($P/P_0 = 0.14$ to 0.30 for **1-meso50**) than that of **1-micro** ($P/P_0 = 0.10$ to 0.17) (fig. S32). This experiment also suggested that the gate-opening pressure can be controlled by the crystal size.

We performed similar experiments on $[\text{Cu}_2(\text{bdc})_2(\text{bpe})]_n$ [2, bpe = 1,2-bis(4-pyridyl) ethylene], which is a twofold interpenetrated framework with larger unit cell parameters than

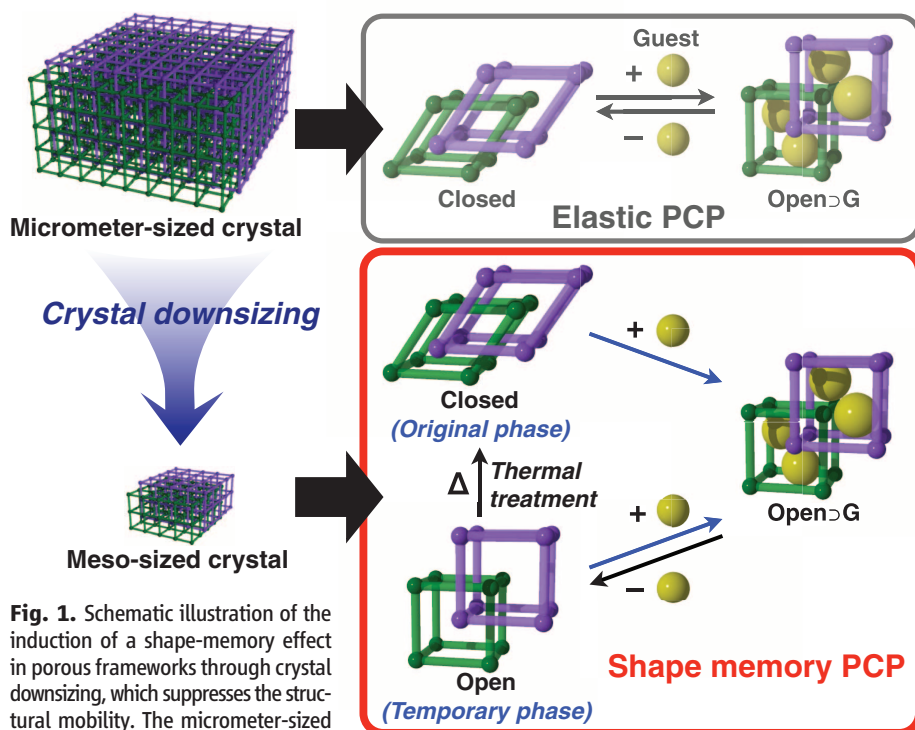


Fig. 1. Schematic illustration of the induction of a shape-memory effect in porous frameworks through crystal downsizing, which suppresses the structural mobility. The micrometer-sized crystals of the two-fold interpenetrated porous frameworks of $[\text{Cu}_2(\text{bdc})_2(\text{bpy})]_n$ (**1**) show the elastic framework flexibility in response to guest incorporation. By downsizing the crystal to the mesoscale, an empty open structure is isolated as an unusual, temporary deformed phase and is then converted to the original closed phase by thermal treatment. This novel structural flexibility is defined as the shape-memory effect in PCPs.

those of **1** (fig. S43 to S56). The open dried phase was isolated in the metastable state when the crystals were downsized to ~ 700 by 700 by 80 nm³ (**2-meso700**) and interconvertible with

the closed phase (figs. S58), which indicated the generation of a MSME. As the crystal size of **2** was decreased further (300 nm or less), the open dried phase could no longer be converted to the

closed phase, even after heating to 200°C (fig. S57). Thus, the smaller crystals of **2-meso300** and **2-meso50** were no longer flexible but had become rigid, indicating a suppression of the structural mobility induced by crystal downsizing. These results suggest that framework flexibility could be controlled by crystal size. The emergence of an unprecedented framework rigidity in the smaller meso-sized crystals of **2** implies that the MSME was induced in the framework as an intermediate phenomenon between the elastic and the rigid frameworks achieved by crystal downsizing.

Regardless of the crystal size, the process of guest removal, which transforms the structure from the guest-included open phase to the closed phase, probably results in the formation of the open dried phase. In the micrometer-sized crystal, this phase was transient and easily converted to the more stable closed phase. Crystal downsizing apparently stabilized the transient state into a metastable state at room temperature. This stabilization is attributed to the thermodynamic and/or kinetic suppression of the phase transition from the open dried phase to the closed phase (fig. S59). The change in the relative thermodynamics between the closed and open dried phases is likely induced by an increase in the surface enthalpy contribution, which has been observed previously for condensed metal oxide systems (20).

However, we observed stabilization in the relatively large crystal size of **2-meso700**, so kinetic suppression should also be considered. Kinetic suppression of the phase transition originates from the barrier between the two phases. We investigated the effect of crystal size on the kinetic suppression using the hysteretic sorption isotherm attributed to the barrier between the closed phase and the open phase (21). The methanol isotherm for **1-meso300** showed a larger

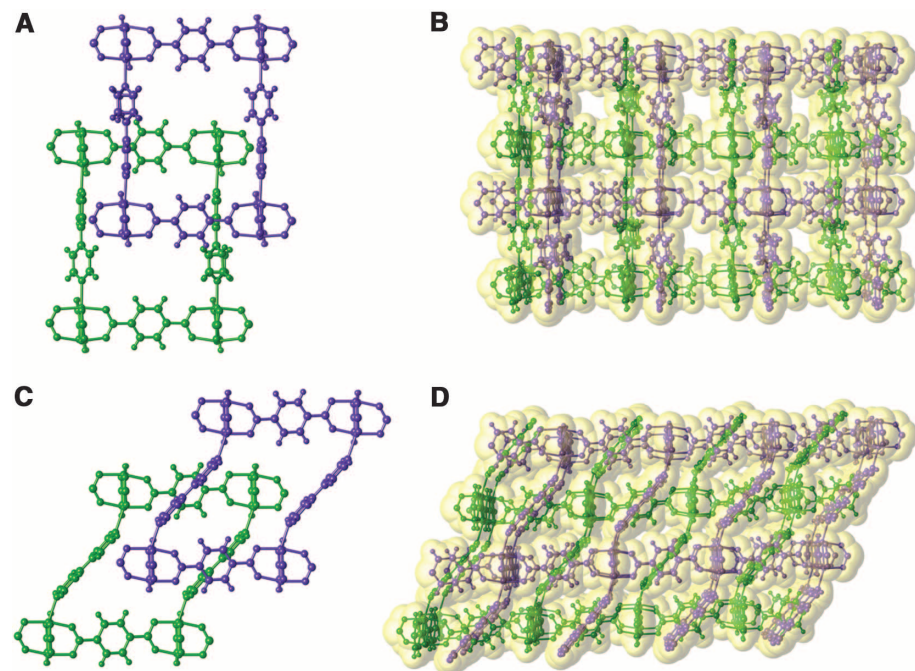


Fig. 2. The structural dynamics of **1** in response to the incorporation of guest molecules. (A and B) The crystal structure of the DMF-incorporated framework of **1**•DMF illustrates the open framework structure: the standard structural unit of interpenetration (A) and the overall framework structure (B) with the view along the main axis of bdc. The guest DMF molecules are omitted for clarity. (C and D) The crystal structure of the guest-removal phase of **1** illustrates the closed framework structure without permanent porosity: the standard structural unit of interpenetration (C) and the overall framework structure (D) with the view along the main axis of bdc. One of the interpenetrated frameworks is highlighted in green and the other in purple. The van der Waals surface is highlighted in yellow.

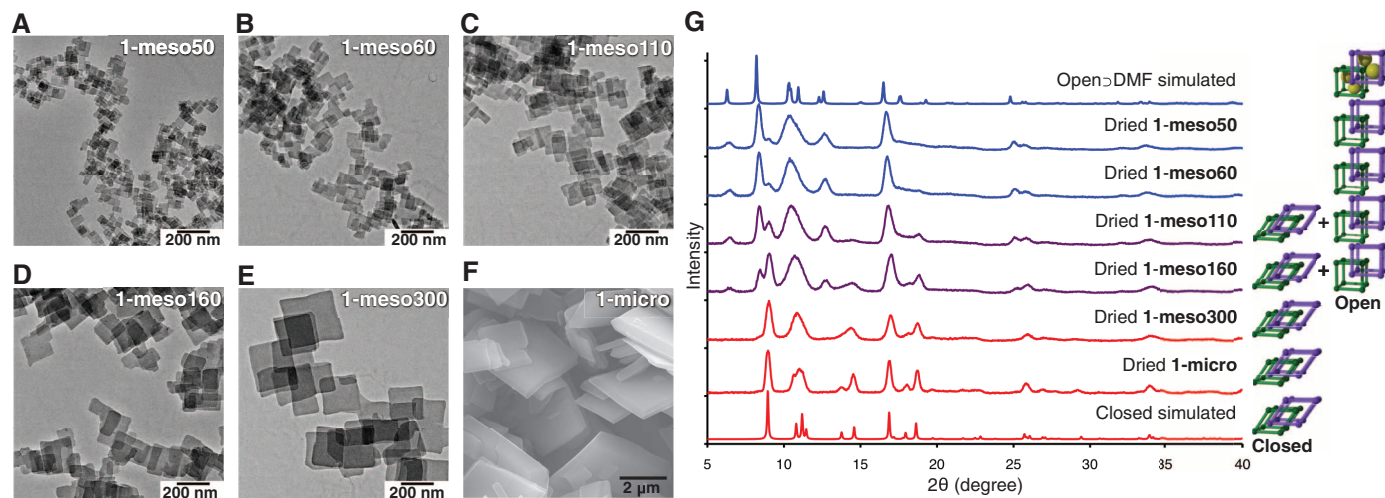


Fig. 3. (A to E) Transmission electron microscopy (TEM) images of meso-sized crystals. (A) **1-meso50**, (B) **1-meso60**, (C) **1-meso110**, (D) **1-meso160**, and (E) **1-meso300**. With an increase in the modulator, the crystal size observed in the TEM image gradually increased. (F) Scanning electron microscopy image of **1-micro**. (G) X-ray diffraction patterns of complete dried crystals of all above-

mentioned sizes, compared with simulated patterns. The closed phase was observed under completely dried conditions for large crystals of **1-micro** and **1-meso300**. With decreasing crystal size, the contribution of the unusual open dried phase increased. The smallest crystal of **1-meso50** was mainly composed of the open dried phase.

hysteretic width compared with that of **1-micro**; however, the center of the hysteric loop was maintained (fig. S60), indicating that the energy barrier of the structural transformation was higher for the smaller crystals. The structural transformation described herein is cooperative and lattice-distortive, which are characteristics of martensitic transformations. The size-dependent kinetic suppression of martensitic materials (22) is often explained by a decrease in the number of lattice defects, which exist even in high-quality crystals. According to the heterogeneous nucleation and growth mechanism of the martensitic phase transformation, the defects provide sites with different nucleation potencies and cooperativity dominates growth (23). Based on the assumption that larger crystals contain more nucleation sites than smaller crystals, a statistical model attributed to the nucleation potency distribution explains the enhanced kinetic suppression of the phase transition by crystal downsizing (fig. S61) (24).

We used the closed and open dried phases as the initial states for the demonstration of switchable adsorption (25). The methanol adsorption isotherm of the open dried phase of **1-meso50** did not show gate-type sorption behavior but

instead presented type I uptake, followed by desorption along the adsorption profile, which is characteristic sorption behavior generally observed in rigid microporous materials (Fig. 4B and fig. S34) (26). As supported by environmentally controlled XRD measurements, structural change did not occur during methanol adsorption (fig. S33). The pore shape was switched from the temporary open dried phase to the original closed phase by heating to 200°C. The resulting material showed adsorption with gate-type sorption behavior (Fig. 4C and fig. S35). The desorption isotherm of the closed phase was similar to that of the open dried phase, indicating that the open dried phase was regenerated after complete desorption. Indeed, the following adsorption isotherm again exhibited a type I profile and could be superimposed over that of the initial open dried phase (Fig. 4D and fig. S36). We further confirmed the repeatability of switching: The adsorption capacity of the open dried phase did not change at all, even after 20 cycles of the open/closed reversible transformation (figs. S37 to S39). We also observed similar adsorption properties (that is, the sorption change between the two distinct initial states) for CO₂ (figs. S40 to S42). The induc-

tion of a MSME for both liquid- and gas-phase molecules could be exploited as intelligent functional materials that respond to the microscopic environmental changes.

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Supplementary Materials

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Materials and Methods

Supplementary Text

Figs. S1 to S61

Table S1

References (27–29)

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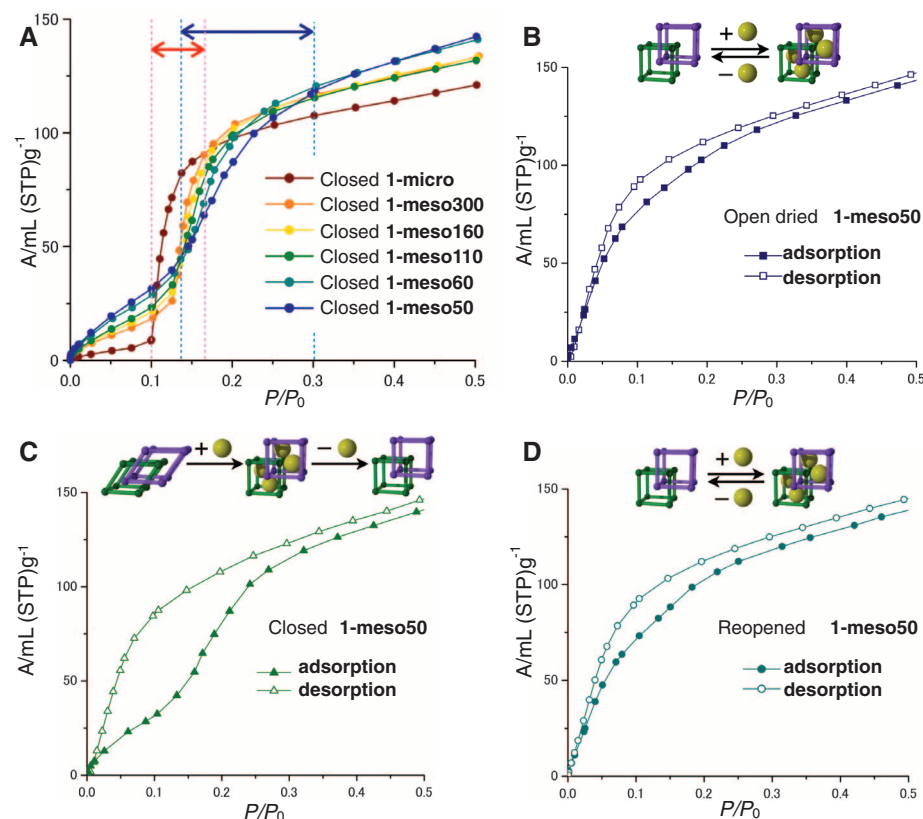


Fig. 4. (A) Methanol adsorption isotherm at 303 K of the closed phase of **1-meso50** (blue), **1-meso60** (turquoise), **1-meso110** (green), **1-meso160** (yellow), **1-meso300** (orange), and **1-micro** (red). Arrows represent the pressure regions in which the structural change from the closed phase to the open phase was observed for **1-micro** (red) and **1-meso50** (blue). (B to D) Switchable methanol adsorption behavior of the shape-memory effect in **1-meso50** (298 K). (B) Isotherm of the open dried **1-meso50**. (C) Isotherm of the closed **1-meso50** generated by heating of the open dried **1-meso50**. (D) Second cycle of the isotherm of the closed **1-meso50** (i.e., isotherm of the reopened **1-meso50**). STP, standard temperature and pressure.

Glutamate-Dependent Neuroglial Calcium Signaling Differs Between Young and Adult Brain

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An extensive literature shows that astrocytes exhibit metabotropic glutamate receptor 5 (mGluR5)–dependent increases in cytosolic calcium ions (Ca^{2+}) in response to glutamatergic transmission and, in turn, modulate neuronal activity by their Ca^{2+} -dependent release of gliotransmitters. These findings, based on studies of young rodents, have led to the concept of the tripartite synapse, in which astrocytes actively participate in neurotransmission. Using genomic analysis, immunoelectron microscopy, and two-photon microscopy of astrocytic Ca^{2+} signaling in vivo, we found that astrocytic expression of mGluR5 is developmentally regulated and is undetectable after postnatal week 3. In contrast, mGluR3, whose activation inhibits adenylate cyclase but not calcium signaling, was expressed in astrocytes at all developmental stages. Neuroglial signaling in the adult brain may therefore occur in a manner fundamentally distinct from that exhibited during development.

The concept that electrically nonresponsive astrocytes can sense glutamatergic transmissions dates to 1990, when it was first described that cultured astrocytes exhibit increases in cytosolic Ca^{2+} in response to glutamate (1). This observation, in combination with the finding that astrocytic Ca^{2+} increases are linked to release of gliotransmitters, forms the basis for the concept of the tripartite synapse (2–4). Ample evidence has supported the observation that excitatory transmission evokes Ca^{2+} signaling in astrocytes by

activation of metabotropic glutamate receptors (mGluRs). For example, stimulation of glutamatergic neuronal afferents in hippocampal slices triggers Ca^{2+} waves in astrocytes inhibited by mGluR5 antagonists (2, 5). However, almost all studies to date have employed either slices prepared from young rodents or cultured astrocytes in their analysis of neuron-glia signaling. Transcriptome analyses suggest that expression of mGluR5 is developmentally regulated and that astrocytic mGluR5 peaks at postnatal day 7 (fig. S1) (6, 7). To assess whether astrocytes express Gq-linked mGluR beyond this young age, we harvested adult human and murine astrocytes using fluorescently activated cell sorting followed by microarray analysis (FACS-array). Adult cortical or hippocampal tissue was dissociated and fluorescently tagged with an antibody directed against the astrocyte-specific glutamate transporter EAAT2 (GLT1) (8, 9). Two

distinct populations of cells, GLT1⁺ (astrocytes) and GLT1[−] (all other cell types), were isolated, and the relative enrichment of genes known to be selectively expressed by astrocytes was evaluated in the two postsorted populations by quantitative real-time fluorescence polymerase chain reaction (qPCR) (fig. S2, A and B). Aldehyde dehydrogenase 1 family member L1 (*ALDH1L1*), the water channel aquaporin 4 (*AQP4*), the excitatory amino acid transporter 2 (*GLT1*, *SLC1A2*), and glial fibrillary acidic protein (GFAP) exhibited a relative enrichment of 20- to 1000-fold in GLT1⁺ cells (fig. S2, C and D). Conversely, the GLT1[−] pool was depleted of genes expressed by other cell types (fig. S2, C and D). The pattern of expression of these genes was confirmed in both human and mouse astrocytes (fig. S2, C and E) (8). Moreover, immunolabeling and qPCR analysis confirmed that *ALDH1L1* and *AQP4* are enriched in the GLT1⁺ pool of cells isolated from 1-, 2-, 3-, and 12-week-old mice (fig. S3, A and B).

We next asked which mGlu receptors are expressed by cortical human astrocytes. The analysis suggested that mGluR3 is the major mGlu receptor expressed by adult cortical human astrocytes, whereas the expression of other mGluRs was low or absent (Fig. 1A). Similar data were collected from murine cortical and hippocampal astrocytes (Fig. 1, B and C). qPCR confirmed that only mGluR3 is enriched in adult human and mouse astrocytes (Fig. 1, D to F). In contrast, mGluR5 is only significantly expressed in astrocytes harvested from 1-week-old mice, with mGluR5 levels falling sharply by the end of week 2 (Fig. 1F) and displaying a continued decline through adulthood (Fig. 1F, inset). To validate isolation of astrocytes in young pups based on their GLT1⁺ expression, we also isolated *Aldh1l1*-eGFP⁺ cells in 1-week-old pups. *Aldh1l1* is the most homogeneously expressed astrocyte marker (10). qPCR analysis showed that both *ALDH1L1*⁺ and GLT1⁺

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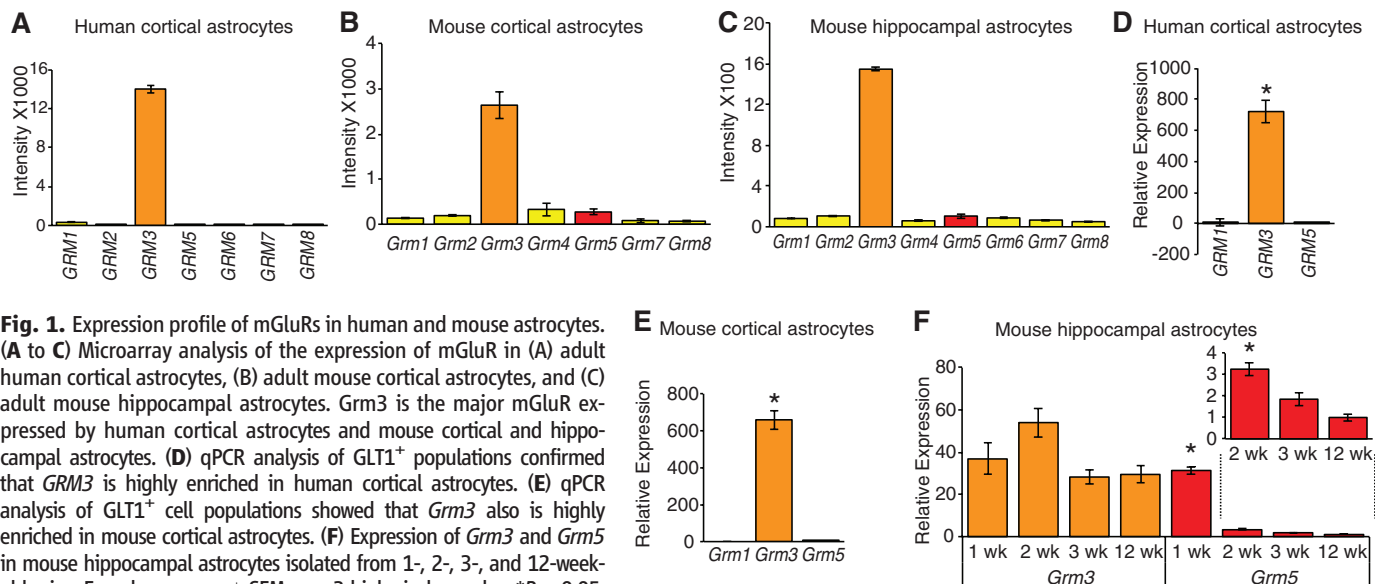


Fig. 1. Expression profile of mGluRs in human and mouse astrocytes. (A to C) Microarray analysis of the expression of mGluR in (A) adult human cortical astrocytes, (B) adult mouse cortical astrocytes, and (C) adult mouse hippocampal astrocytes. *Grm3* is the major mGluR expressed by human cortical astrocytes and mouse cortical and hippocampal astrocytes. (D) qPCR analysis of GLT1⁺ populations confirmed that *GRM3* is highly enriched in human cortical astrocytes. (E) qPCR analysis of GLT1⁺ cell populations showed that *Grm3* also is highly enriched in mouse cortical astrocytes. (F) Expression of *Grm3* and *Grm5* in mouse hippocampal astrocytes isolated from 1-, 2-, 3-, and 12-week-old mice. Error bars, mean $\pm$ SEM; $n = 3$ biological samples. * $P < 0.05$, one-way ANOVA, Bonferroni multiple comparisons test.

cells' populations in 1-week-old pups exhibited comparable levels of mGluR3 and mGluR5 mRNA expression (fig. S3C).

To further validate our observations of the expression of mGluR5 and mGluR3 in developing astrocytes, we immunostained cortical and

hippocampal tissue of adult mice with mGluR5 or mGluR2/3 antibodies and examined the relative distribution of labeling across different

Fig. 2. Electron micrographic analysis of mGluR2/3 and mGluR5 in the adult mouse cortex and hippocampus. (A to D) Electron micrographs showing examples of mGluR5-immunoreactive elements, and (E to G) mGluR2/3-immunoreactive elements in the mouse hippocampus (Hipp) and cerebral cortex (Ctx). Ax, unmyelinated axons; Den, dendrites; As, astrocytes; Te, axon terminals. Scale bars, 0.5 μ m. (H and I) Histograms showing the relative percentage of mGluR5- or mGluR2/3-immunoreactive elements categorized as presynaptic (terminals and unmyelinated axons) or postsynaptic (dendrites and spines) neuronal elements or glia. For each region and antibody, data were collected in three mice. A total of 2215 μ m² of mGluR5 or mGluR2/3 immunostained tissue was examined in both cortex and hippocampus.

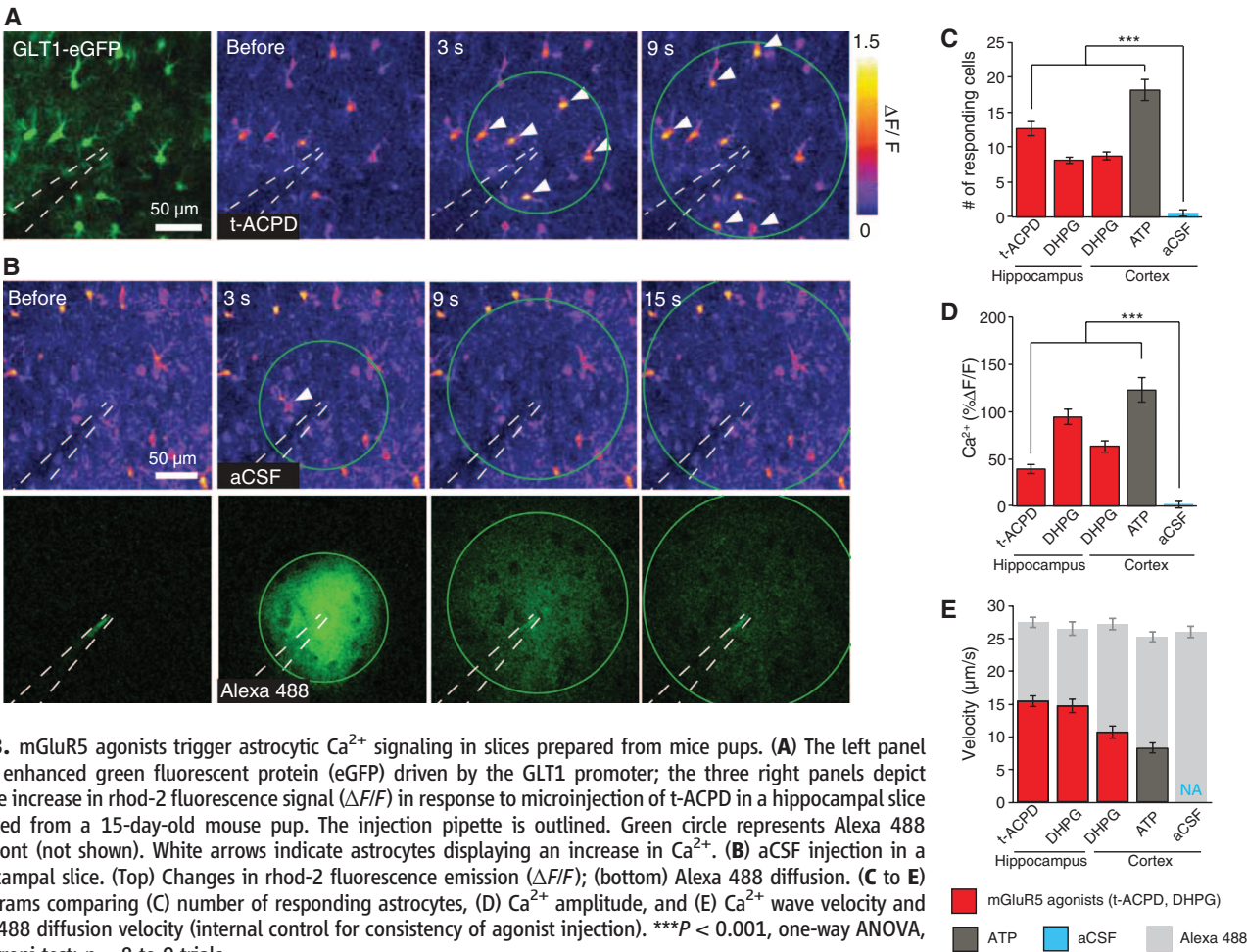
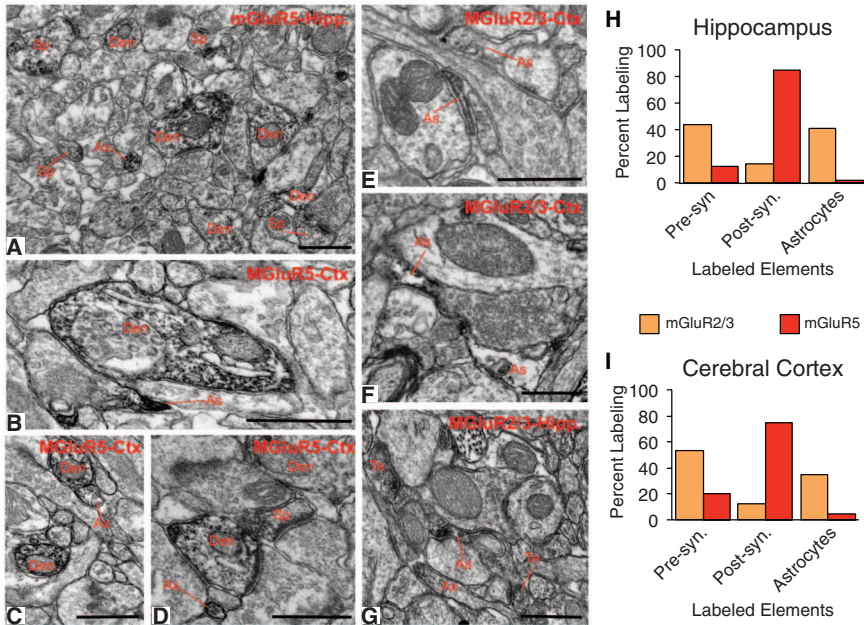


Fig. 3. mGluR5 agonists trigger astrocytic Ca²⁺ signaling in slices prepared from mice pups. (A) The left panel shows enhanced green fluorescent protein (eGFP) driven by the GLT1 promoter; the three right panels depict relative increase in rhod-2 fluorescence signal ($\Delta F/F$) in response to microinjection of t-ACPD in a hippocampal slice prepared from a 15-day-old mouse pup. The injection pipette is outlined. Green circle represents Alexa 488 wavefront (not shown). White arrows indicate astrocytes displaying an increase in Ca²⁺. (B) aCSF injection in a hippocampal slice. (Top) Changes in rhod-2 fluorescence emission ($\Delta F/F$); (bottom) Alexa 488 diffusion. (C to E) Histograms comparing (C) number of responding astrocytes, (D) Ca²⁺ amplitude, and (E) Ca²⁺ wave velocity and Alexa 488 diffusion velocity (internal control for consistency of agonist injection). *** $P < 0.001$, one-way ANOVA, Bonferroni test; $n = 8$ to 9 trials.

neuronal and glial elements identified by their respective ultrastructural features (11). In both the cerebral cortex and hippocampus, the overall pattern of labeling for the two receptor subtypes was very similar (i.e., 70 to 80% immunoreactivity for mGluR5 was associated with postsynaptic elements such as spines and dendritic shafts), whereas less than 5% labeled structures were accounted for by glial processes (Fig. 2, A to D, H, and I). In contrast, labeling of group II mGluRs was rarely encountered in postsynaptic structures but was frequently seen in astrocytic processes (35 to 45% labeling) and preterminal axonal segments (Fig. 2, E to I) (12, 13).

To compare the efficacy by which group I mGluR1/5 Gq-coupled receptors mobilize intracellular Ca^{2+} stores in young and adult mice, we used two-photon imaging to quantify astrocytic Ca^{2+} signaling in response to two mGluR5 agonists: 3,5-dihydroxyphenylglycine (DHPG) and ($\pm$)-1-aminocyclopentane-*trans*-1,3-dicarboxylic acid (t-ACPD). Acute hippocampal and cortical slices were prepared from mice pups on postnatal (P)

days P12 to P15 and loaded with rhod-2 acetoxymethyl ester (AM) (Fig. 3A). The agonists were microinjected with a pipette filled with artificial cerebrospinal fluid (aCSF) containing Alexa 488 (100 μM) (Fig. 3B). Pressure injection of vehicle (aCSF) was adjusted to trigger Ca^{2+} increases in 0 to 2 cells per field (Fig. 3, B and C). DHPG (200 μM) or t-ACPD (500 μM) triggered robust Ca^{2+} waves in both hippocampal and cortical astrocytes (Fig. 3, C to E, and fig. S4). Adenosine triphosphate (ATP) (500 μM) also evoked robust increases in astrocytic Ca^{2+} (Fig. 3, C to E).

We next asked whether mGluR1/5 agonists also induced Ca^{2+} signaling in vivo (Fig. 4, A and B). The agonists were microinjected into the cortex of ketamine/xylazine-anesthetized pups (P12 to P15) or adult mice. Cortical astrocytes in mice pups displayed potent increases in Ca^{2+} in response to mGluR5 agonists (Fig. 4C), which were directly comparable to observations in acute cortical slices (Fig. 4, D and E). When the same experiments were repeated in adult anesthetized mice, astrocytes failed to respond to the mGluR5

agonists, DHPG or t-ACPD, as well as (RS)-2-chloro-5-hydroxyphenylglycine (CHPG, 500 μM). The number of astrocytes responding to either of these mGluR5 agonists was not statistically different from control aCSF injections (Fig. 4, C to E, and figs. S5 and S6). Analysis with electroencephalography showed that t-ACPD or DHPG did not significantly alter local neuronal activity (fig. S7). Because anesthesia can suppress astrocytic Ca^{2+} signaling (14, 15), t-ACPD injections were repeated in awake, nonanesthetized mice. Microinjection of t-ACPD in awake mice triggered an increase in Ca^{2+} in an average of 0.67 ± 0.33 cells ($n = 7$ trials), which is not significantly different from either vehicle control or t-ACPD injection in anesthetized mice [analysis of variance (ANOVA), Bonferroni test]. Application of the Gi-coupled mGluR3 receptor agonists, *N*-acetylaspartylglutamate (NAAG, 1 mM) or LY379268 (100 μM) failed, as expected, to elicit astrocytic Ca^{2+} signaling in the mouse brain (Fig. 4, C and D). The lack of agonist-induced response did not reflect an inability of adult astrocytes to mobilize cytosolic

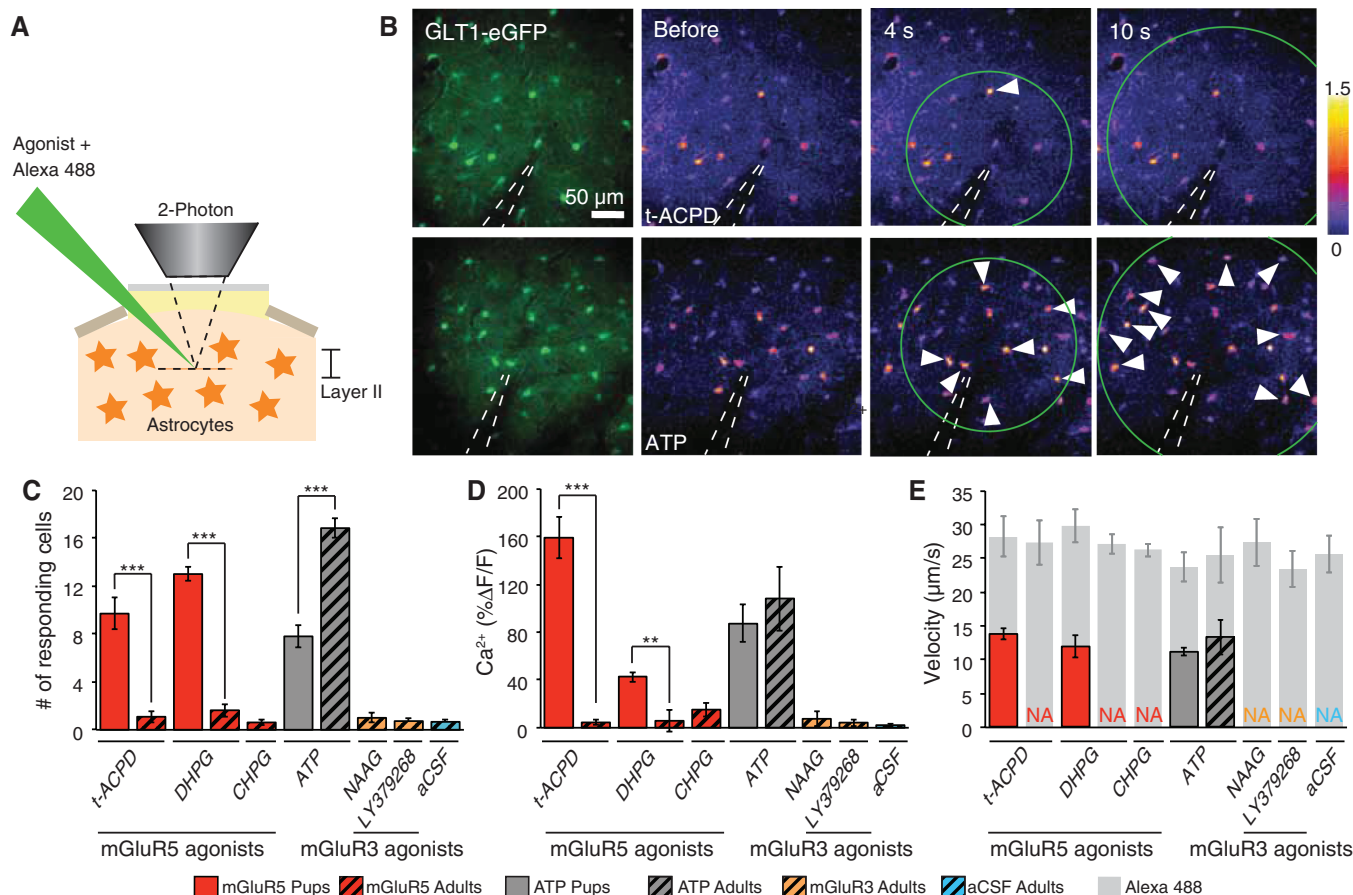


Fig. 4. Multiple mGluR5 agonists fail to trigger astrocytic Ca^{2+} signaling in adult mice. (A) Experimental setup used for microinjection of agonist in cerebral cortex layer II in anesthetized mice loaded with rhod-2 AM. (B) (Top left) EGFP driven by the astrocyte-specific GLT1 promoter in an adult mouse. (Top right) Time lapse of changes in rhod-2 fluorescence signal ($\Delta F/F$) in response to microinjection of t-ACPD (500 μM). One astrocyte (white arrow) exhibited an increase in rhod-2 signal. (Bottom) Rhod-2 signal changes ($\Delta F/F$)

in response to ATP (500 μM) in an adult mouse. White arrows indicate astrocytes that exhibited an increase in Ca^{2+} . (C to E) Histograms comparing (C) number of astrocytes displaying an increase in Ca^{2+} in response to agonist injections in pups and adult mice, (D) Ca^{2+} amplitude, and (E) Ca^{2+} wave velocity and Alexa 488 diffusion velocity (internal control for consistency of agonist injection). ** $P < 0.01$, *** $P < 0.001$, one-way ANOVA, Bonferroni test; $n = 4$ to 19 trials.

Ca^{2+} because ATP potently induced Ca^{2+} increases (Fig. 4C).

The tripartite synapse forms a central element in work implicating astrocytes in long-term potentiation and complex neurocognitive functions such as sleep (2, 5, 16, 17). Our analysis has broad implications because it shows that Gq-coupled group I mGluRs are not expressed by adult murine cortical and hippocampal astrocytes or by adult cortical human astrocytes. Astrocytic Ca^{2+} signaling evoked by synaptic release of glutamate may thus be confined to the young rodent pups, which most commonly are employed in studies of neuron-glia signaling (3). The observations reported here do not call into question that astrocytes can be indirectly activated by neural activity. A number of transmitters, including endocannabinoids, purines, norepinephrine, and acetylcholine, as well as changes in extracellular Ca^{2+} , can trigger astrocytic Ca^{2+} signaling (14, 18–21). Yet, activation of these pathways is typically limited to episodes of intense glutamatergic transmission or to the global release of neuromodulators that occur in the setting of arousal or awakening.

Our analysis shows that adult astrocytes express mGluR3, which is a Gi/Go receptor negatively coupled to adenylate cyclase. Can activation of mGluR3 trigger gliotransmitter release? This seems unlikely, because mGluR3 agonist failed to trigger Ca^{2+} increases in astrocytes (Fig. 4, C and

D), and regulated exocytosis generally is enhanced by cyclic adenosine monophosphate (cAMP)–dependent protein kinase (PKA). The slow time scale of Gi/Go-coupled processes likely excludes their involvement in rapid synaptic events. Thus, the existing literature plus the lack of mGluR5-induced Ca^{2+} increases suggest that mGluR3 receptors are not involved in gliotransmitter release.

In particular, our observations indicate that the Gi/Go-coupled suppression of cAMP signaling is the principal intracellular pathway by which astrocytes monitor local glutamatergic transmission in the adult CNS and further suggest that glutamatergic signaling per se is insufficient to trigger astrocytic Ca^{2+} signaling.

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Supplementary Materials

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Materials and Methods
Figs. S1 to S7
References (22–25)

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Neural Basis of a Pollinator's Buffet: Olfactory Specialization and Learning in *Manduca sexta*

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Pollinators exhibit a range of innate and learned behaviors that mediate interactions with flowers, but the olfactory bases of these responses in a naturalistic context remain poorly understood. The hawkmoth *Manduca sexta* is an important pollinator for many night-blooming flowers but can learn—through olfactory conditioning—to visit other nectar resources. Analysis of the flowers that are innately attractive to moths shows that the scents all have converged on a similar chemical profile that, in turn, is uniquely represented in the moth's antennal (olfactory) lobe. Flexibility in visitation to nonattractive flowers, however, is mediated by octopamine-associated modulation of antennal-lobe neurons during learning. Furthermore, this flexibility does not extinguish the innate preferences. Such processing of stimuli through two olfactory channels, one involving an innate bias and the other a learned association, allows the moths to exist within a dynamic floral environment while maintaining specialized associations.

Floral scent mediates a range of plant-pollinator associations, from specialized interactions (1–3) to those that are more generalized through associative learning of the scent and nectar reward by the pollinator (1, 4–7). Although it is generally assumed that many flower species may have converged on a similar scent profile to attract a specific pollinator or pollinator class, identification of the compounds mediating the innate responses and how learning alters the behavior

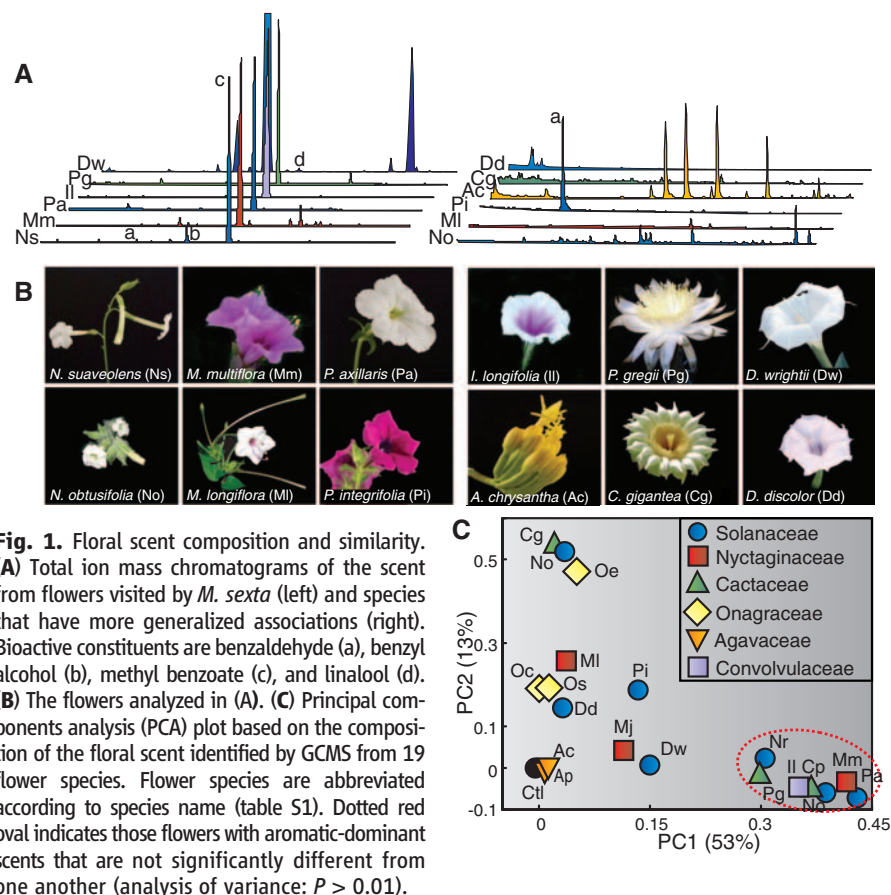
remain unclear. The hawkmoth *Manduca sexta* has a wide geographic distribution and is an important pollinator for many night-blooming plants, which make it an excellent system in which to explore the neural basis of olfactory specialization and learning. *M. sexta* has an innate olfactory preference for certain night-blooming plants that exhibit characteristics typical of moth-pollinated flowers (for example, *Datura wrightii* flowers). The moths, however, also have the ability to learn,

through olfactory conditioning, to utilize other floral resources (5, 8–10).

We characterized the floral scents of plant species that we and others have observed to be visited and pollinated by *M. sexta* moths (supplementary materials, table S1). To help control for phylogenetic differences between plant species, we also characterized the floral scents of related plant species that have more generalized pollinator associations. We collected floral volatile organic compounds (VOCs) using dynamic sorption methods and analyzed the trapped mixtures by gas chromatography with linked mass spectrometry (GCMS) (11, 12). Although many of the hawkmoth-visited flowers differed qualitatively and quantitatively in their scent profiles, their scents were often dominated by oxygenated aromatic compounds (35 to 90% of the total floral headspace VOCs), especially methyl benzoate, benzyl alcohol, and benzaldehyde (Fig. 1A). By contrast, flowers that are visited by other pollinator taxa were dissimilar in their scents in comparison with the hawkmoth-visited flowers (Fig. 1). Moreover, similarity in scent composition between species

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did not group according to phylogenetic relatedness (Fig. 1C), which suggested that the flowers may have converged on a similar sensory signal to attract hawkmoths.

To gain insight into the representation of floral scents in the olfactory system of the moth, we conducted electrophysiological recording experiments on the moth's antenna and in the antennal (olfactory) lobe (AL). When the ipsilateral antenna was stimulated with VOCs from the aromatic-rich flowers, projection (output) neurons in the anterior region of the AL responded strongly (Fig. 2A). Moreover, single-unit and neural-ensemble responses to the hawkmoth-preferred floral VOCs were not significantly different from one another (Fig. 2, B and C; Kruskal-Wallis test: $P = 0.69$), but were significantly different from ensemble responses to the VOCs of flowers visited by other pollinator taxa (Fig. 2, B and F; Kruskal-Wallis test: $P < 0.001$). Further recordings in the periphery and AL showed that oxygenated aromatics elicited the strongest responses and that the moth-visited flower species were processed similarly (figs. S1 and S2). These findings suggest a common olfactory percept of the aromatic-rich flower species even when the scents differed in composition and complexity.

But does olfactory response necessarily reflect behavioral preference by the moths? To answer this question, we performed behavioral experiments with naïve moths. Moths were exposed, one at a time, to an array of visually identical artificial

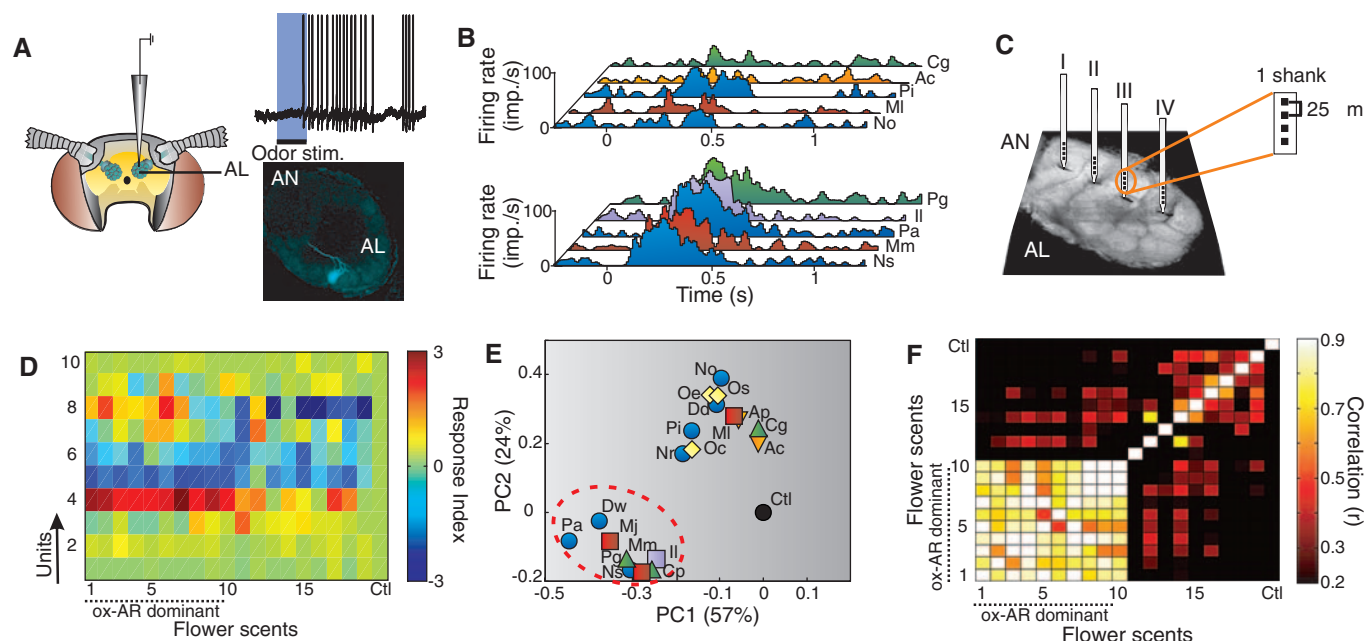


Fig. 2. Antennal-lobe neuronal response to floral scents. (A) Intracellular morphology and recordings of PN responses to scents from hawkmoth-visited flowers. AL, antennal lobe; AN, antennal nerve. (B) PN responses to scents from flowers not visited by *M. sexta* (top) and hawkmoth-visited flowers (bottom). (C) Confocal-microscopic image of AL showing the locations of the recording sites of the multichannel array used to record the

neural ensemble responses. (D) Ensemble response to floral scents. (E) PCA of the firing-rate responses of the ensemble of neurons to the different floral scents. The red oval indicates not significantly different responses to hawkmoth-visited floral scents (Kruskal-Wallis test: $P > 0.10$). (F) Correlation coefficients between ensemble responses to the different flower scents.

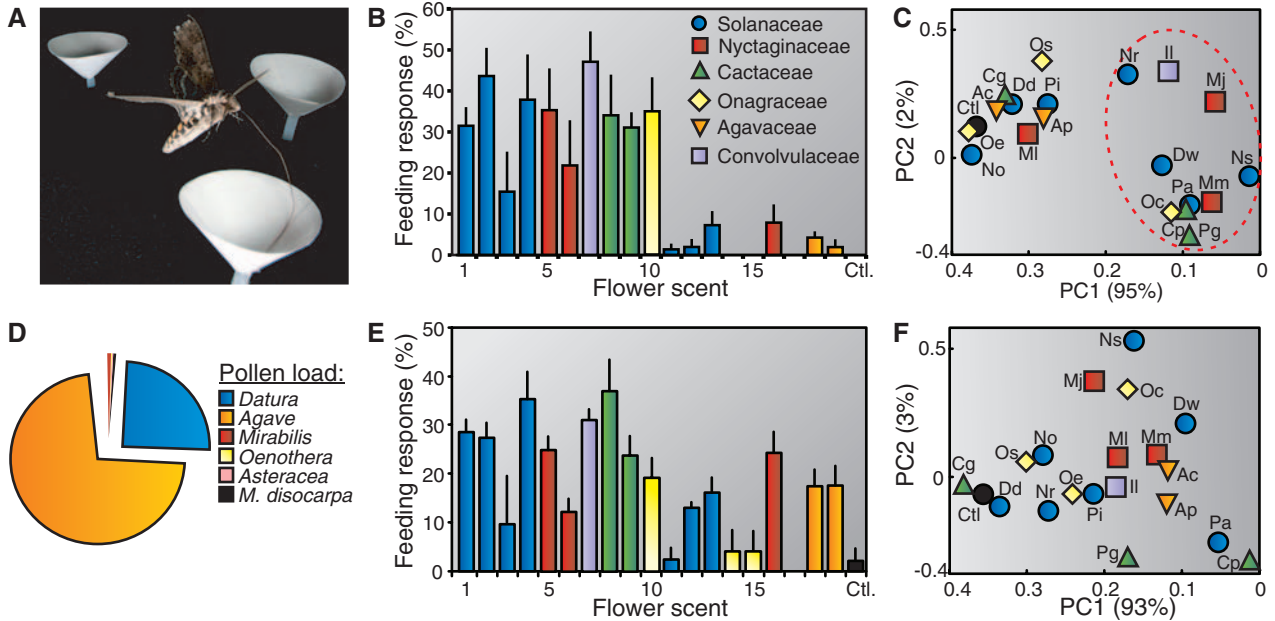


Fig. 3. (A) Image of a *M. sexta* moth attempting to feed from one in an array of 20 artificial flowers used to test the attractiveness of different floral scents. **(B)** Feeding responses by lab-reared *M. sexta* to the different floral scents. **(C)** PCA plot of the behavioral responses (table S3) to the different scents. Dashed

red oval indicates those hawkmoth-visited floral scents that were not significantly different ($P > 0.05$). **(D)** Pollen species found on the proboscises of wild *M. sexta*. **(E and F)** Wild *M. sexta* feeding responses (E) and PCA plot of behavioral responses (F) to the different floral scents.

(paper) flowers, each emitting the VOC mixture from a different floral species (Fig. 3A). Results of these experiments showed that naïve moths attempted to feed from flowers emitting the aromatic-rich scents, and the naïve moths visited these flowers at approximately similar frequencies (Fig. 3B; two-by-two χ^2 test: $P > 0.05$). By contrast, scents of the majority of floral species that lacked the oxygenated aromatic compounds elicited significantly lower visitation and feeding rates (Fig. 3B; two-by-two χ^2 test: $P < 0.002$). Thus, the scents of hawkmoth-visited flowers were processed, and perceived, similarly by the moths to drive innate behavior.

A fundamental component of pollinator behavior is the flexibility to switch among floral resources on the basis of the pollinator's ability to learn (7, 13), but how learning shapes the innate responses of pollinators remains unexplored. We therefore took advantage of the association between *M. sexta* moths and bat-adapted *Agave palmeri* flowers that occurs in the semi-arid Sonoran Desert of southern Arizona (5, 14). *M. sexta* moths can learn to feed from the bat-adapted *A. palmeri* flowers by associating its abundant nectar reward with its floral scent, which is not innately attractive to moths (5). When wild moths that had previous experience with *Agave*, *Datura*, and *Mirabilis* spp. were exposed to the flower array, moths responded to those specific floral scents, but like naïve animals, they also responded to the other flowers with the aromatic-rich scents (Fig. 3, E and F). For more controlled examination of the effects of prior experience with flowers on moth preferences, we used laboratory-reared moths that had learned to feed from *A. palmeri* flowers

(5). As with wild moths, *A. palmeri*-experienced moths also attempted to feed from flowers emitting *Agave* VOCs and from the hawkmoth flowers (table S5). Thus, olfactory conditioning provided moths with flexibility in their foraging behavior but did not extinguish their innate preferences for scents from the moth-pollinated flowers.

To determine how the olfactory system processes odor information as the moth learns, we performed experiments involving simultaneous multichannel AL recording and olfactory conditioning (Fig. 4A and fig. S3) (15). This approach allowed us to correlate the neural and behavioral responses directly as the animal learned the association between the *A. palmeri* scent and sugar reward (fig. S3) (16, 17). After three training trials, the moths exhibited significantly increased proboscis-movement responses (PMRs) from 0 to 33% (χ^2 test: $P < 0.01$). The ability to learn the scent association was directly correlated with the change in AL neural responses evoked by the VOCs (Fig. 4B). As the moths learned, the firing rate of AL neurons significantly increased, response latency decreased, firing synchrony between neurons increased, and the pattern of neural activity by the ensemble changed significantly (Fig. 4 and fig. S4; Kruskal-Wallis test: $P < 0.02$). By contrast, moths that were not rewarded or for which the reward was paired randomly with VOC stimulation had significantly lower PMRs than the trained moths and did not learn the association (0% PMR for both treatments; χ^2 test: $P < 0.001$). Furthermore, AL neuronal activity in the control moths did not change significantly over the course of the experiment (fig. S4; Kruskal-Wallis test with multiple comparisons: $P \geq 0.10$). Last, dif-

ferential conditioning experiments were conducted to determine how learning influences the coding of a scent paired with a reward (CS+; *A. palmeri*) versus an unpaired scent (CS−; *Carnegiea gigantea*). These experiments demonstrated that moths could readily learn to discriminate between the two complex scents and that modulation of the AL ensemble served to increase the difference in representation of the two scents (fig. S5; Kruskal-Wallis test: $P < 0.05$). Thus, learning the association between an ecologically important floral scent and sugar-water reward significantly modified AL neural responses and increased the contrast between scents.

The change in the AL associated with learning leads to the question of how this plasticity occurs. Biogenic amines—in particular, octopamine and dopamine—have been shown in other insects to participate in the acquisition of olfactory memory and modulation of AL neural circuitry (18–21). Because putative receptors for octopamine and dopamine are expressed in the *M. sexta* AL (10, 22), we injected receptor antagonists or agonists into the AL before training the moths to associate the *A. palmeri* VOCs with a sugar reward. Results of these experiments demonstrated that microinjection of dopamine-receptor antagonists {fluphenazine [10^{-8} M], Le300 [10^{-6} M], or SCH23390 [10^{-6} M]} had no effect on the ability of moths to associate the reward with the *A. palmeri* odor and were not significantly different from the results obtained with control moths (two-by-two χ^2 test: $P \geq 0.55$). By contrast, injection of octopamine-receptor antagonists {epinastine or mianserine [10^{-6} M]} prevented the moths from learning the odor-reward association and elicited significantly lower PMRs

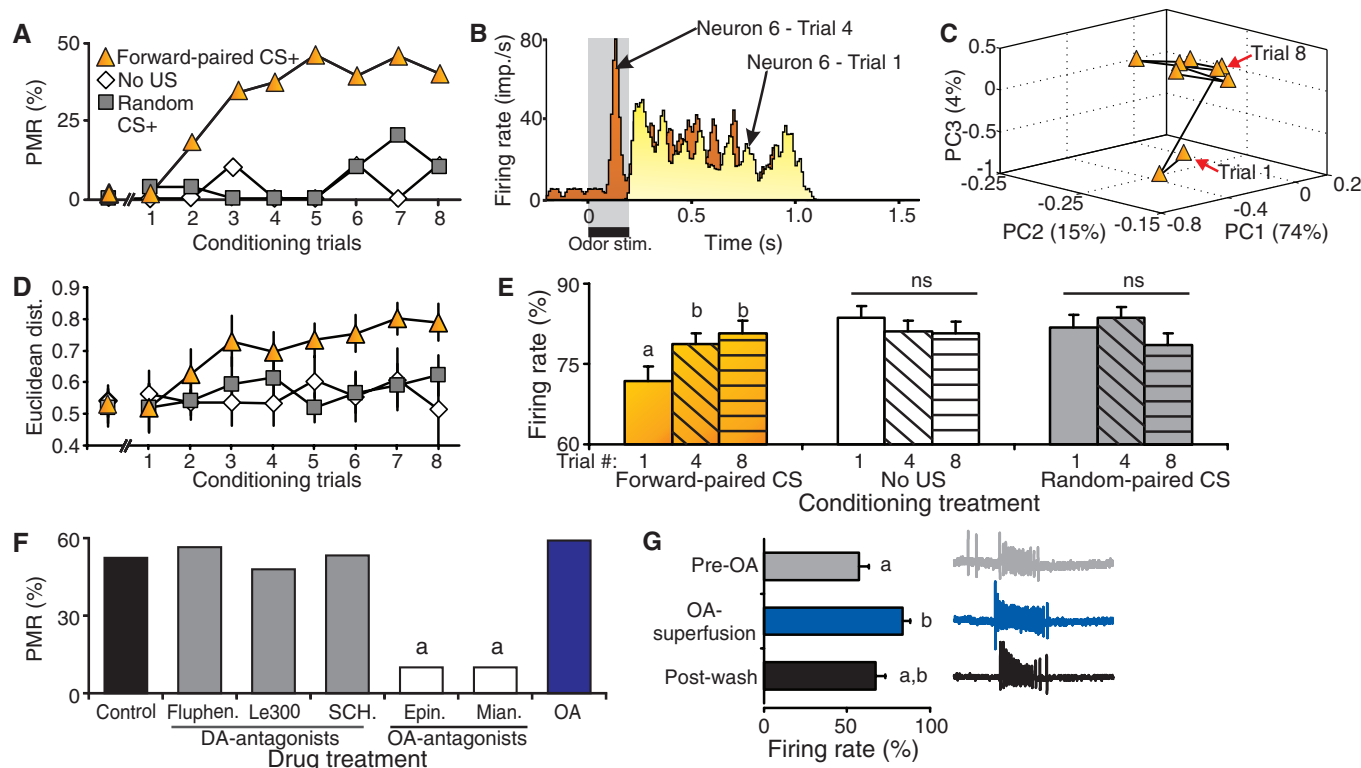


Fig. 4. Olfactory learning modifies the neural representation of the scent in the AL. (A) Olfactory learning during classical forward-paired conditioning experiments with *A. palmeri* scent and sucrose reward (orange triangles). Moths did not learn the association in the absence of the unconditioned stimulus (white diamonds), or the random paired conditioned stimulus (CS)—unconditioned stimulus (US) treatment (gray circles). (B) Response of an AL neuron to stimulation with *A. palmeri* scent at trial 1 (yellow) and trial 4 (orange). (C) PCA plot of the ensemble responses (e.g., PC1) to the *A. palmeri* scent through training. (D) Change in Euclidean distance of the ensemble representation of the *A. palmeri* scent during training. (E) Change in firing rate of AL neurons during trials 1, 4 (diagonal bars), and 8 (hashed bars) for moths in the different conditioning treatments: forward-paired (orange bars), no-US reward (white bars), and random-paired (gray bars). (F) In individual moths, focal microinjection of receptor antagonists [dopamine-receptor antagonists (DA) or octopamine-receptor antagonists (OA)], and agonist (OA) were conducted before conditioning. (G) Superfusion of octopamine significantly increased the firing rate of PN responses. Letters denote a significant difference between treatments ($P < 0.05$).

compared with the vehicle control (Fig. 4F; two-by-two χ^2 test: $P < 0.01$). Moreover, when octopamine (at 10^{-5} M) was injected into the AL and moths were stimulated with the VOCs without a paired sugar reward, these moths exhibited significantly higher PMRs in reaction to the VOC stimulus than did moths that were injected with the vehicle (Fig. 4F; two-by-two χ^2 test: $P < 0.001$). Finally, superfusion of octopamine (10^{-5} or 10^{-6} M) onto the AL during multichannel and juxtacellular neural recordings elicited effects similar to those observed when the moth learned. Neurons showed increased VOC-evoked firing rates, response latencies decreased, and firing synchrony between neurons increased (Fig. 4G and fig. S6). Thus, octopamine apparently acts as a key neuromodulator in the moth's olfactory pathway to mediate interactions with flowers in the field.

When considering the effects of learning on innate preferences, it is important to examine the strength or evolutionary importance of the association. Our results show that learning does not extinguish responses to the innately attractive floral scents and, thus, suggest that olfactory conditioning may operate in an olfactory “channel”

separate from, but parallel to, that involved in the innate responses. In the case of *M. sexta*, many, but not all, of the flowers that elicit innate responses are also solanaceous plants that are host plants for ovipositing females. In such cases, the flower may signal an appropriate host or a site at which to locate a mate, and thus, the strength of the association may be a function of the important life-history events that are unrelated to the immediate foraging responses of the moths. However, some of the flowers that emit the aromatic-rich scents that elicit innate responses are not host plants, which raise the question of how these diverse species have evolved similar floral scents to attract hawkmoths. We here provide impetus for such future work by showing that moths perceive these diverse floral species similarly.

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Supplementary Materials

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Materials and Methods

Figs. S1 to S7
Tables S1 to S5
References (23–36)

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Ezh2 Orchestrates Topographic Migration and Connectivity of Mouse Precerebellar Neurons

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We investigated the role of histone methyltransferase *Ezh2* in tangential migration of mouse precerebellar pontine nuclei, the main relay between neocortex and cerebellum. By counteracting the sonic hedgehog pathway, *Ezh2* represses *Netrin1* in dorsal hindbrain, which allows normal pontine neuron migration. In *Ezh2* mutants, ectopic *Netrin1* derepression results in abnormal migration and supernumerary nuclei integrating in brain circuitry. Moreover, intrinsic topographic organization of pontine nuclei according to rostrocaudal progenitor origin is maintained throughout migration and correlates with patterned cortical input. *Ezh2* maintains spatially restricted *Hox* expression, which, in turn, regulates differential expression of the repulsive receptor *Unc5b* in migrating neurons; together, they generate subsets with distinct responsiveness to environmental *Netrin1*. Thus, *Ezh2*-dependent epigenetic regulation of intrinsic and extrinsic transcriptional programs controls topographic neuronal guidance and connectivity in the cortico-ponto-cerebellar pathway.

In mammals, cortical motor and sensory information is mostly relayed to the cerebellum via the hindbrain precerebellar pontine nuclei (PNs), which include pontine gray and reticulotegmental nuclei. The developing hindbrain is rostrocaudally segregated into progenitor compartments, or rhombomeres (r1 to r8) (1), genetical-

ly defined by nested *Hox* gene expression (2). Mouse PN neurons are generated from r6 to r8 lower rhombic lip progenitors (3), undergo a long-distance caudorostral tangential migration via the anterior extramural stream (AES), and settle beside the ventral midline (Fig. 1, A and B) (4, 5). Intrinsic expression of transcription factors and

guidance receptors and extrinsic distribution of ligands are important for AES migration (6–8). However, little is known about the epigenetic regulation of these transcriptional programs. Here, we addressed the role of *Ezh2*, which is member of the Polycomb repressive complex 2 and trimethylates histone H3 at lysine 27 (H3K27me3) (9).

Ezh2 transcripts are maintained through late stages in lower rhombic lip progenitors, migratory stream, and PN neurons (fig. S1), whereas H3K27m3 is detected throughout the hindbrain (fig. S2). To conditionally inactivate *Ezh2*, we generated transgenic lines in which *Cre* is driven by rhombomere-specific enhancers in spatially restricted regions tiling the caudal hindbrain (10) (figs. S3 and S4). To assess cell-autonomous and/or region-specific non-cell autonomous *Ezh2* function in pontine neuron migration, we first crossed *Krox20::Cre* (10) to an *Ezh2*^{fl/fl} allele (10) (*Krox20::Cre;Ezh2*^{fl/fl}). Inactivation in r3

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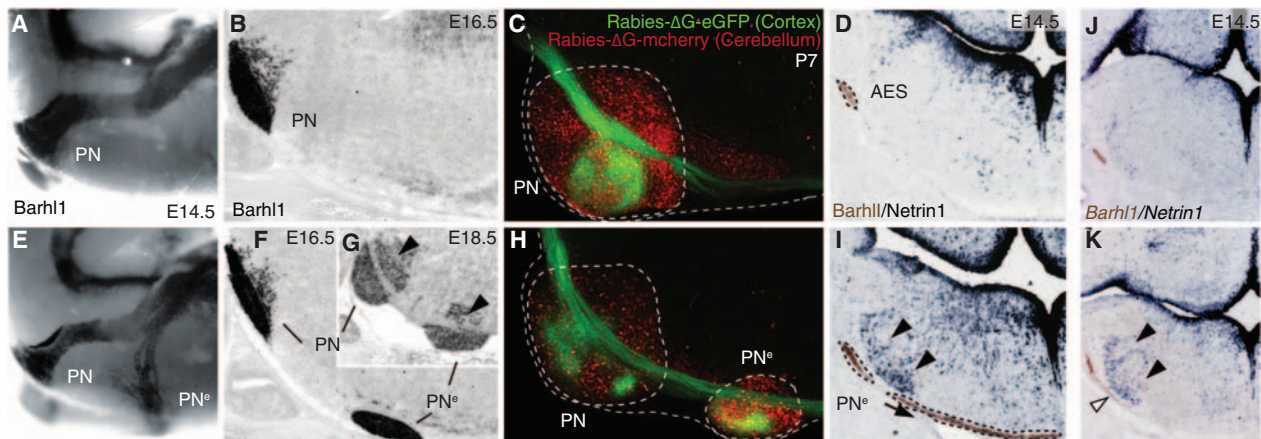


Fig. 1. *Ezh2* non-cell autonomous role in pontine neuron tangential migration. (A, B, E, F, and G) Migratory phenotypes in control (A) and (B) and *r5-6::Cre;Ezh2*^{fl/fl} mutants (E) to (G). *Barhl1* in situ hybridization in E14.5 whole-mount (A) and (E), E16.5 (B) and (F), and E18.5 (G) sagittal sections. Pontine gray and reticulotegmental [arrowheads (G)] nuclei (PNs) are duplicated (PN^es). (C and H) Tracrings from P7 cortex (rabies-ΔG-eGFP) and cerebellum

(rabies-ΔG-mCherry) in controls (C) and *r5-6::Cre;Ezh2*^{fl/fl} mutants (H). PNs and PN^es are connected to cortex and cerebellum. (D, I, J, and K) *Barhl1/Netrin1* expression in E14.5 control (D), *r5-6::Cre;Ezh2*^{fl/fl} (I), *r5post::Cre;Ezh2*^{fl/fl}; *Shh*^{fl/+} (J), and *r5post::Cre;Ezh2*^{fl/fl}; *Shh*^{fl/fl} (K) coronal sections. Ectopic *Ntn1* [arrowheads: (I) and (K)] and PN^e ectopic migration [arrow and white arrowhead, respectively: (I) and (K)] are partially rescued (J).

and r5, which do not contribute to the pontine migratory stream (3), resulted in small ectopic PNs in posterior r5 (PN^cs) (fig. S5), which supports an *Ezh2* non-cell autonomous role. Deletion in r5 and r6 (*r5-6::Cre;Ezh2^{fl/fl}*) resulted in a more prominent phenotype. A neuronal subset split from the migratory stream, turned ventrally, and generated an ectopic duplication of PNs (PN^cs) (Fig. 1, E to G). PN^c neurons were nonrecombined *Ezh2^{+/+}* H3K27me3⁺ located within the r5- and r6-derived territory mostly devoid of H3K27me3 (fig. S2), which confirmed *Ezh2*'s non-cell autonomous function.

To assess whether PN^cs integrated cortico-cerebellar connectivity, we carried out cortex-to-PN and cerebellum-to-PN tracings. We injected viral constructs expressing green fluorescent protein (GFP) (rabies-ΔG-GFP) and/or mCherry (rabies-ΔG-mCherry) in postnatal day 2 (P2) wild type and *r5-6::Cre;Ezh2^{fl/fl}* and *Krox20::Cre;Ezh2^{fl/fl}* mutants. At P7, PNs and PN^cs triggered collateralization of corticospinal axons and innervated the cerebellum (Fig. 1, C and H, and fig. S6).

To evaluate *Ezh2* cell-autonomous function, we used the *Wnt1::Cre* deleter (10). *Ezh2* transcripts and H3K27me3 were selectively deleted from lower rhombic lip and migratory stream in *Wnt1::Cre;Ezh2^{fl/fl}* mutants (figs. S2 and S7). Nonetheless, the mutation was not sufficient to induce ectopic posterior pontine neuron migration (Fig. 2, J and M, and figs. S5 and S7). The most severe phenotype was observed in *Hoxa2::Cre;Ezh2^{fl/fl}* mutants, where *Ezh2* was inactivated in both AES neurons and their migratory environment, i.e., throughout r3- to r6- and dorsal r7- to r8-derived structures including the lower rhombic lip (figs. S2 and S3). The whole AES did not migrate anterior to r6 and settled into a single posterior ectopic nu-

cleus (PN^c) (fig. S5). Thus, *Ezh2* has a non-cell autonomous role in AES migration, which is enhanced by a cell-autonomous function in progenitors and migrating neurons.

Netrin1 (Ntn1) and Slit1-3 are attractive or repulsive secreted cues that influence pontine neuron migration (6, 7). *Slit1-3* expression was not altered in embryonic day 14.5 (E14.5) *r5-6::Cre;Ezh2^{fl/fl}* mutants (fig. S8). In contrast, *Ntn1*, normally expressed in floor-plate and ventral ventricular progenitors (Fig. 1D and fig. S5) (11), was ectopically expressed in dorsal progenitors and the mantle layer medially to the AES in *r5-6::Cre;Ezh2^{fl/fl}*, *Krox20::Cre;Ezh2^{fl/fl}*, *Hoxa2::Cre;Ezh2^{fl/fl}*, and *r5post::Cre;Ezh2^{fl/fl}* mutants (Fig. 1I and fig. S5). Additional deletion of *Shh* was sufficient to prevent strong ectopic *Ntn1* activation in dorsal progenitors of E12.5 *r5post::Cre;Ezh2^{fl/fl}*; *Shh^{fl/fl}* conditional mutants (fig. S5). At E14.5, ectopic *Ntn1* was almost undetectable and the *Ezh2* knockout was partially rescued (Fig. 1, J and K, and fig. S5). Therefore, *Ezh2* is required to restrict *Ntn1* expression to ventral progenitors by silencing *Ntn1* in the dorsal neural tube. However, *Ezh2* deletion is not sufficient to ectopically induce *Ntn1*, which additionally requires *Shh* signaling from the floor plate. Thus, in the dorsal neural tube, *Ezh2*-mediated epigenetic repression of *Ntn1* may normally counteract *Shh*-mediated activation.

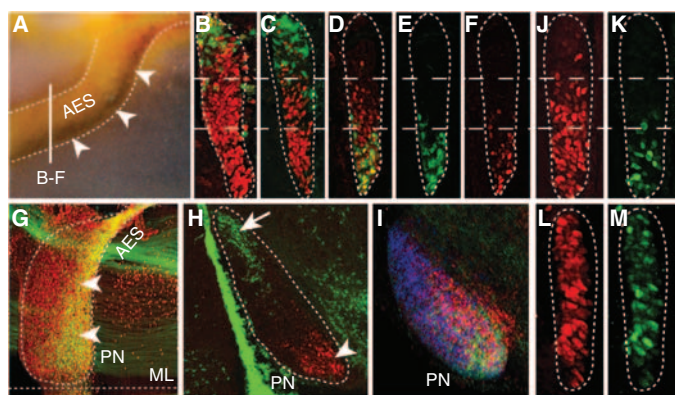
Ectopic and/or increased environmental Ntn1 levels may trigger premature migration toward the midline. In E14.5 *r5-6::Cre;Ezh2^{fl/fl}* mutants, only a subset of pontine neurons split from the stream and entered the alternative ventral migratory pathway at the level of the ectopic *Ntn1⁺* domain (Fig. 1I and fig. S5), which suggested that AES neurons may display intrinsic differential

responsiveness to Ntn1 signaling. To map the contributions of r6 (*r6RLP*) or r7 and r8 (*r7-8RLP*) lower rhombic lip-derived neuronal progenies into the pontine stream and nuclei (Fig. 2, B, C and H, and fig. S3), we crossed floxed reporter lines to *r5-6::Cre* or *r7post::Cre* (*Cre* is expressed up to the r6-r7 boundary) (fig. S3H) in which *Cre* is down-regulated before AES migration (fig. S4). *r6RLP* mapping was confirmed by the tamoxifen-inducible *MafB::CreERT2* transgenic line, whose reporter expression pattern is restricted to r5 and r6, similar to that in *r5-6::Cre* (10) (fig. S3). To trace the whole precerebellar lower rhombic lip progeny (*r6-8RLP*), we used *r5post::Cre* (figs. S3A and S4).

r6-8RLP contributed to the whole PNs (fig. S3), whereas *r6RLP* mapped to the most anterior (arrow in Fig. 2H and fig. S3), and *r7-8RLP* filled the remaining posterior portions of PNs (fig. S3). This topographic organization of pontine neuronal subsets directly correlated with their relative position within the migratory stream. Namely, *r6RLP* mapped to the dorsalmost AES, whereas *r7-8RLP* contributed to the remaining portion ventrally to *r6RLP* (Fig. 2, B and C). Thus, the precerebellar lower rhombic lip is rostrocaudally mapped onto the AES dorsoventral axis (Fig. 3E and fig. S1J) and, in turn, onto the PN rostrocaudal axis (fig. S1K), with neuronal subsets maintaining their relative position throughout migration and settling.

Next, we investigated molecular correlates of this intrinsic cellular regionalization and asked whether *Hox* paralog groups (PG) 2 to 5 maintain their spatially restricted progenitor expression patterns in pontine migratory stream and nuclei (Fig. 2). Indeed, *Hox* PG2 (*Hoxa2/Hoxb2*) and PG3 (*Hoxa3/Hoxb3*), expressed in the whole precerebellar rhombic lip, were correspondingly maintained throughout the pontine migratory stream and nuclei (6) (fig. S1). *Hoxb4* is normally expressed up to the r6-r7 boundary, whereas the *Hox* PG5 rostral expression limit is posterior to PG4 genes (2). In the AES and PNs, *Hoxb4⁺* neurons extended just ventrally and posteriorly, respectively, to *r6RLP* (Fig. 2, C and D, and fig. S1L), whereas *Hoxa5* and *Hoxb5* transcripts and *Hoxa5* protein mapped to the ventralmost migratory stream and posteriormost PNs, respectively (Fig. 2, A, F, H and I, and figs. S1 and S2). Simultaneous detection of *Hoxa5* and ZsGreen in *r5-6::Cre;R26R^{ZsGreen}* specimens demonstrated rostrocaudal segregation of *r6RLP* and *Hoxa5⁺* neurons within the PNs (Fig. 2H). To permanently label *Hoxa5*-expressing neurons, we generated a transgenic line in which *Cre* was inserted in-frame at the *Hoxa5* locus (*Hoxa5::Cre*) (10) (fig. S3). *Hoxa5::Cre*-expressing neurons segregated to the ventralmost AES and posteriormost PNs, faithfully overlapping endogenous *Hoxa5* distribution (Fig. 2, D, E, F and G, and figs. S1F, S3, and S4). Thus, pontine neuron subsets of distinct rostrocaudal origin maintain their relative topographic positions and *Hox* codes throughout migration and settling within the target nucleus (Fig. 4A and fig. S1).

Fig. 2. Intrinsic topography of pontine migratory stream and PNs and *Ezh2*-dependent *Hox* regulation. (A) *Hoxb5/Barhl1* in situ hybridization on E15.5 whole-mount brain (lateral view). Arrowheads show *Hoxb5⁺* neuron ventral restriction in AES. (B and C) E15.5 *r5-6::Cre;R26R^{ZsGreen}* AES coronal sections costained with ZsGreen and Pax6 (red) (B) or ZsGreen and *Hoxb4* (red) (C) showing complementary dorsal ZsGreen⁺ and ventral *Hoxb4⁺* cell distributions [bar in (A) shows section level]. (D to F) E15.5 *Hoxa5::Cre;R26R^{ZsGreen}* AES sections showing partially overlapping ZsGreen and *Hoxb4* (red) costainings with offset dorsal limits (D); ZsGreen⁺ (E) and *Hoxa5⁺* (F) neurons display similar ventral restriction. (G and H) E15.5 whole-mount *Hoxa5::Cre;R26R^{ZsGreen}* (G) or *r5-6::Cre;R26R^{ZsGreen}* (H) sagittal sections costained with ZsGreen and Pax6 (red) or ZsGreen and *Hoxa5* (red), respectively, illustrating posterior PN restriction of *Hoxa5⁺* neurons [arrowheads: (G) and (H)], and anterior restriction of r6-derived neurons [arrow (H)]. ML, ventral midline. (I) *Hoxb3/Hoxb4/Hoxb5* nested in situ expression patterns on PN sagittal section (J to M) *Hoxb4* (red) and *Hoxa5* (green) immunostaining of E15.5 *Wnt1::Cre;Ezh2^{fl/fl}* control (J) and (K) and *Wnt1::Cre;Ezh2^{fl/fl}* mutant (L) and (M) AES. In (L) and (M), *Hoxb4* and *Hoxa5* lose their spatial restriction and are ectopically derepressed up to the dorsal edge of the AES.



Is *Ezh2* required to maintain *Hox* nested expression in migrating pontine neurons and PN^s? In E14.5 *Wnt1::Cre;Ezh2^{fl/fl}* and *Hoxa2::Cre;Ezh2^{fl/fl}* mutants, *Hoxb4*, *Hoxa5*, and *Hoxb5* were ectopically expressed within the anterior lower rhombic lip and spread ventrodorsally throughout the

pontine migratory stream (Fig. 2, L and M, and figs. S2 and S7). Thus, by preventing *Hox PG4* and *PG5* expression in anterior precerebellar rhombic lip and migrating neuronal progeny, *Ezh2*-mediated repression contributes to the maintenance of molecular heterogeneity in the migratory stream.

This, in turn, may underlie intrinsic differential response of migrating neuron subsets to environmental Ntn1.

Netrin-mediated attraction is counteracted by *Unc5* repulsive receptors (12), and *Unc5c* inactivation results in variable ectopic migration of AES

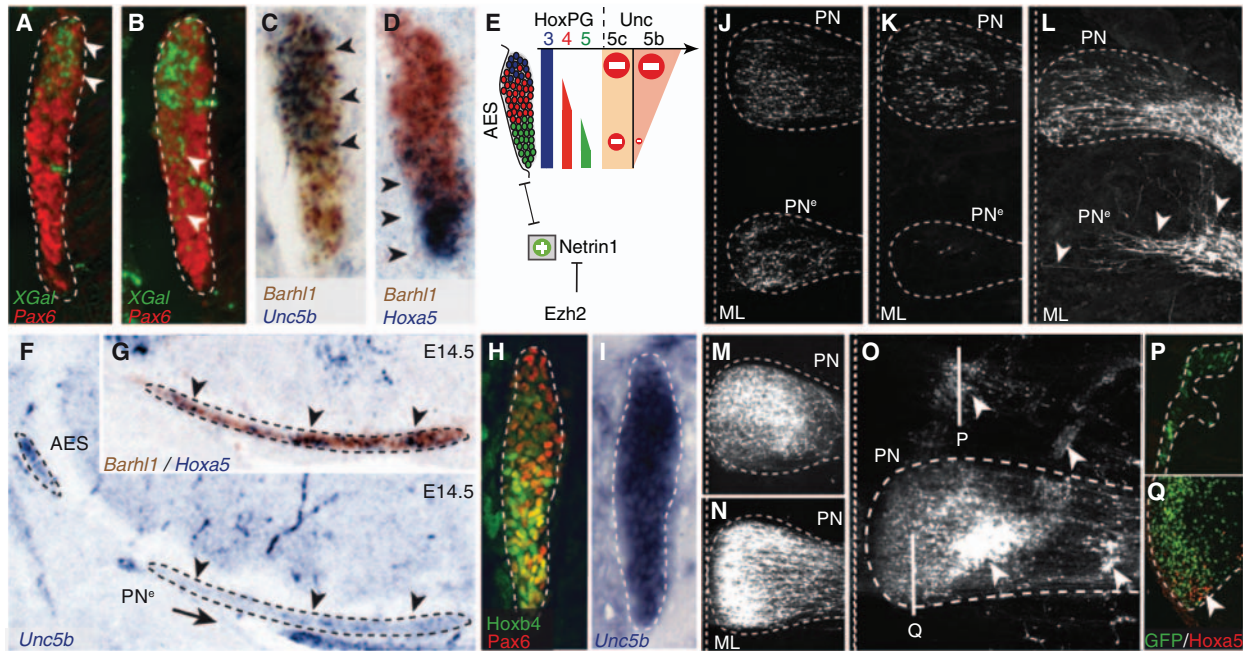
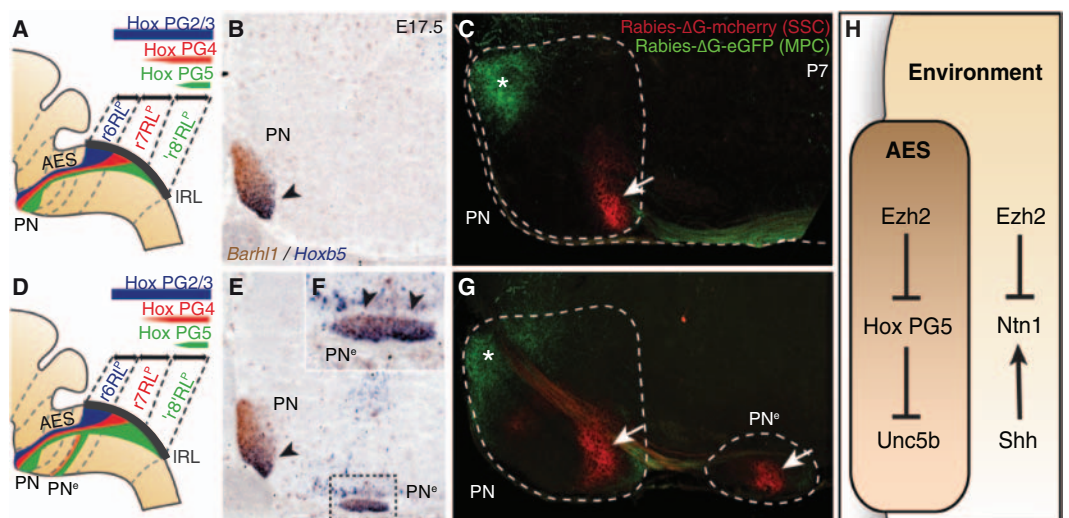


Fig. 3. *Ezh2*- and *Hox*-dependent regulation of *Unc5b* in pontine neuron migration. (A and B) X-gal (green) and Pax6 (red) stainings of E14.5 *Unc5b^{bGal/+}* heterozygotes (A) and *Unc5b^{bGal/bGal}* homozygotes (B) showing X-gal-stained cell distribution in AES (arrowheads). (C to E) *Unc5b/Barhl1* (C) and *Hoxa5/Barhl1* (D) in situ hybridization in E14.5 AES showing complementary dorsoventral expression of *Unc5b* and *Hoxa5* (arrowheads) and summary (E). (F and G) In *r5-6::Cre;Ezh2^{fl/fl}* mutants, PN^e migrating neurons are *Unc5b*-negative (F) and *Hoxa5⁺/Barhl1⁺* (G) (arrowheads). (H and I) In E14.5 *Hoxa5^{-/-}/Hoxb5^{-/-}/Hoxc5^{-/-}* AES, *Unc5b* is up-regulated ventrally (I), whereas *Hoxb4* and *Pax6* are normally expressed (H). (J and K) In utero EP in

E14.5 *r5-6::Cre;Ezh2^{fl/fl}* mutants of *Unc5b/Unc5c/eGFP* strongly reduces at E18.5 ectopically migrating PN^e neurons (K), as compared with EP of eGFP (J), and partially rescues the phenotype. ML, ventral midline. (L to Q) In E13.5 wild type, EP of *Ntn1* results in posterior ectopic pontine neuron migration at E17.5, phenocopying *r5-6::Cre;Ezh2^{fl/fl}* mutants [arrowheads (L)]. Although EP at E13.5 of eGFP (M) or *Unc5c/eGFP* constructs has no apparent effect on migration at E18.5 (N), EP of *Unc5b/eGFP* results in anterior ectopic migration and/or dorsal-lateral arrest [arrowheads (O)]. Immunostaining on sagittal sections shows that anterior ectopic GFP+/Unc5b+ electroporated cells are *Hoxa5*-negative (P); *Hoxa5⁺* cells are normally restricted in posterior PN (Q).

Fig. 4. PN regionalization and patterned cortical input. (A, B, D, E, and F), *Hox* expression summary in migrating pontine neurons of control (A) and *r5-6::Cre;Ezh2^{fl/fl}* mutants (D). *Barhl1/Hoxb5* in situ hybridization on E17.5 sagittal sections (B), (E), and (F). In *r5-6::Cre;Ezh2^{fl/fl}* mutants (E) and (F), *Hoxb5⁺* neurons spread throughout the rostrocaudal extent of the ectopic nuclei (PN^s) [arrowheads (F)], whereas, in PNs, they are normally posteriorly restricted as in control [arrowheads: (B) and (E)]. (C and G) Rabies-ΔG viruses injected in control visual/medioposterior cortex (MPC) and SSC anterogradely trace fibers into anterior (green,*) and posterior (red, arrow) PNs (C), respectively. In *r5-6::Cre;Ezh2^{fl/fl}* mutants (G), PN^e lacks innervation by MPC, whereas it is innervated by SSC (arrow). (H) *Ezh2*- and *Hox*-dependent genetic circuitry of intrinsic and extrinsic *Unc5b/Ntn1* regulation.



neurons (13). *Unc5c* is expressed in lower rhombic lip progenitors, down-regulated in migrating neurons, and reactivated upon approaching the midline (fig. S9) (13). Thus, *Unc5c* is unlikely to confer a dorsoventrally biased response of the AES to Ntn1. *Unc5b* has been involved in vascular development (14), though a role in neuronal development was not explored. We found a dorsoventral high-to-low density of cells expressing *Unc5b* (Fig. 3C and fig. S9) and β -galactosidase activity in *Unc5b*^{bGal/+} fetuses (Fig. 3A). *Unc5b* expression was, in turn, down-regulated when the migratory stream turned toward the midline (fig. S9C). Dorsoventral *Unc5b* transcript distribution in the migratory stream anti-correlated with *Hox PG5* expression (Fig. 3, C and D). In E14.5 *Hoxa5*^{-/-}; *Hoxb5*^{-/-}; *Hoxc5*^{-/-} compound mutants, *Unc5b* was up-regulated in ventral *Hoxb4*⁺/*Pax6*⁺ AES neurons (Fig. 3, H and I). Thus, *Hox PG5* normally represses *Unc5b* in ventral AES neurons originating from posterior precerebellar lower rhombic lip.

In E14.5 *Unc5b*^{bGal/bGal} null mutants, β -galactosidase⁺ cells partially lost their normal dorsal restriction and spread into ventral AES (Fig. 3B). Thus, *Unc5b* contributes to maintaining topographical organization of dorsal AES subsets. In E16.5 *Unc5c*^{-/-} fetuses, dorsal *Unc5b*-expressing AES neurons maintained their normal migratory path, whereas ectopic neurons were *Hox PG5*⁺ and mainly *Unc5b*-negative (fig. S9). Therefore, in the absence of *Unc5c*, ventral *Unc5b*-negative AES neurons become more sensitive to Ntn1-mediated attraction than dorsal *Unc5b*-expressing neurons. Similarly, in *r5-6::Cre;Ezh2*^{fl/fl} mutants, *Unc5b*-expressing neurons remain dorsal and pursue their normal migration, whereas *Hox PG5*⁺/*Unc5b*-negative neurons are preferentially influenced by Ntn1 up-regulation and ectopically attracted to the midline (Fig. 3, F and G, and fig. S2). Moreover, in *Hoxa2::Cre;Ezh2*^{fl/fl} mutants, in which all pontine neurons are *Ezh2*^{-/-}/*H3K27me3*⁻ and migrate through an environment ectopically expressing *Ntn1* (fig. S5), all migrating neurons are prematurely attracted to an ectopic posterior midline position, are *Hox PG5*⁺, and down-regulate *Unc5b* (figs. S2 and S7).

Next, *Unc5b/5c* overexpression by in utero electroporation (EP) of E14.5 lower rhombic lip progenitors was sufficient to cell-autonomously rescue the PN^c phenotype in *r5-6::Cre;Ezh2*^{fl/fl} mutants [enhanced GFP⁺-positive (eGFP⁺) neuron quantification in PN^cs compared with PN^s: eGFP (*n* = 5) 33.79% $\pm$ 0.1060; eGFP/*Unc5b/5c* (*n* = 5) 0.64% $\pm$ 0.0044; *P* = 0.00011] (Fig. 3, J and K), which demonstrated that elevating *Unc5* receptor levels counteracts increased Ntn1-mediated attraction. Ntn1 overexpression by EP in E13.5 wild-type fetuses induced ectopic posterior migration of AES neurons (Fig. 3L), partially phenocopying the *r5-6::Cre;Ezh2*^{fl/fl} mutant phenotype and showing that increasing Ntn1 is sufficient to cause ectopic ventral migration of neuronal subsets.

Furthermore, although overexpression of *Unc5c* in E13.5 wild-type fetuses had no apparent effect on AES migration (Fig. 3, M and N), *Unc5b* EP

triggered ectopic anterior migration and/or a block in dorsal position of *Hoxa5*-negative pontine neuron subsets (Fig. 3, O to Q). Therefore, maintaining constitutively high *Unc5b* levels in migrating neurons prevents or delays turning toward the midline; the latter results in ectopic anterior migration. Conditional *Hoxa2* overexpression in rhombic lip derivatives by mating *Wnt1::Cre* with a *ROSA26::(lox-STOP-lox)Hoxa2*-internal ribosome entry site (*IRES*)-eGFP (*Wnt1::Cre;R26R*^{*Hoxa2*}) allele (10) also resulted in anterior ectopic migration generating rostrally elongated PN^s, which maintained high *Unc5b* expression, unlike in control mice (fig. S9). Therefore, although *Hox PG5* are involved in negatively regulating *Unc5b* in the ventral migratory stream, *Unc5b* expression in dorsal AES may be under *Hox PG2*-positive regulation and generates differential responses to environmental Ntn1.

Finally, we investigated whether the PN and PN^c patterning differences in *r5-6::Cre;Ezh2*^{fl/fl} mutants result in distinct cortical inputs. In *P7 Pcp2::Cre;R26R*^{*tdTomato*} animals (10) expressing *Cre* in medioposterior (including visual) cortex (MPC), *tdTomato*⁺ axons projected onto the rostral PN, in agreement with (15), including the *r6RLP* neuron subset (fig. S6). Coinjection of rabies- Δ G-GFP and rabies- Δ G-mCherry into visual and/or MPC and medial somatosensory cortex (SSC) resulted in rostral GFP⁺ and caudal mCherry⁺ axonal inputs onto the PN^s, respectively (Fig. 4C). In *r5-6::Cre;Ezh2*^{fl/fl} mutants, PN was targeted both by visual or MPC-derived GFP⁺ (rostrally) and SSC-derived mCherry⁺ (caudally) axons, whereas PN^c was innervated by SSC-derived though not visual/MPC-derived axons (Fig. 4G), correlating with their posterior *Hox PG5*⁺ profile (Fig. 4, A, B, and D to F).

During radial migration, correlation to rostrocaudal position of origin is maintained through interaction with glial progenitors (16). How long-range tangentially migrating neurons (17) maintain information about their origin is less well understood. We show that the topographic migratory program of r6- to r8-derived pontine neurons is largely established in progenitor pools according to rostrocaudal origin and maintained in migrating neurons. We found similar organizational principles during lateral reticular nucleus migration (fig. S10). Moreover, the r2 to r5 rhombic lip also gives rise to neurons that migrate tangentially along a short dorsoventral extramural path and populate distinct brainstem cochlear nuclei with a rostrocaudal topography (3). On its caudorostral route, the precerebellar stream migrates ventrally to the cochlear stream (3), although they do not mix despite close cellular proximity, which suggests that rhombomere-specific programs may control appropriate precerebellar neuron position during tangential migration. Indeed, we show that the topography of r6 versus r7 versus r8 origin is preserved throughout migration, mapped along the dorsoventral axis of the pontine stream, and eventually within rostrocaudal subregions of the PN^s, correlating with patterned

cortical input. The transcriptional regulation of this tangential migratory program is epigenetically maintained (Fig. 4H). *Ezh2*-mediated repression maintains dorsoventrally restricted environmental distribution of attractive and/or repulsive cues, such as *Ntn1*, and an intrinsically heterogeneous *Hox* transcriptional program in the migratory stream that, in turn, provides neuronal subsets with distinct *Unc5b*-dependent responses to environmental Ntn1, and thus contributes to maintaining the neuronal position during migration.

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Supplementary Materials

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Multiple Fitness Peaks on the Adaptive Landscape Drive Adaptive Radiation in the Wild

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The relationship between phenotype and fitness can be visualized as a rugged landscape. Multiple fitness peaks on this landscape are predicted to drive early bursts of niche diversification during adaptive radiation. We measured the adaptive landscape in a nascent adaptive radiation of *Cyprinodon* pupfishes endemic to San Salvador Island, Bahamas, and found multiple coexisting high-fitness regions driven by increased competition at high densities, supporting the early burst model. Hybrids resembling the generalist phenotype were isolated on a local fitness peak separated by a valley from a higher-fitness region corresponding to trophic specialization. This complex landscape could explain both the rarity of specialists across many similar environments due to stabilizing selection on generalists and the rapid morphological diversification rate of specialists due to their higher fitness.

Adaptive radiation, the rapid evolution of ecological and phenotypic diversity within a clade, may account for much of life's diversity (1, 2). The ecological theory of adaptive radiation is founded on the unifying concept of the adaptive landscape, the topographical relationship between fitness and a continuous phenotypic space (1). This theory predicts early bursts of niche diversification due to rapid invasion of multiple, unoccupied fitness peaks after colonization, the evolution of a key innovation, or mass extinction (1–5). This “early burst” model is supported by microbial evolution (4), the fossil record (6), and species diversification in some clades (7), but the pattern is rare in comparative data (8). Indeed, observations of disruptive selection suggest that many populations are constrained from ascending fitness peaks (9, 10).

Most studies of selection estimate its local form—directional, stabilizing, or disruptive (1, 9)—but few investigate the broader topography of the fitness surface, particularly among multiple species (11–13) or traits (14), or by manipulating phenotypes to measure the fitness of intermediates between species (12, 15–17). However, measurement of the broader multivariate fitness landscape is necessary to demonstrate a local maximum and to visualize the selective surface during early bursts of niche diversification. Multipeak fitness landscapes have been demonstrated in laboratory microcosms (4) and inferred from resource availabilities (18), foraging performance (13), and mark-recapture (11) in the field, but not from fitness measurements of manipulated phenotypes. Thus, the key early burst prediction of multiple fitness peaks has never been experimentally tested in the wild.

We measured fitness landscapes directly from growth and survival of F_2 hybrids from crosses among the three species in a sympatric adaptive radiation of *Cyprinodon* pupfishes on San Salvador Island, Bahamas. F_2 hybrids spanned the range of morphological diversity represented in the three parental species. This small island radiation contains ecologically novel species and displays rapid morphological diversification rates similar to those of classic adaptive radiations (19), yet is less than 10,000 years old (20). The endemic radiation contains two trophic specialist species, a scale-eater and a hard-shelled-prey specialist (durophage), both novel ecological niches within *Cyprinodon*, and a third generalist species similar to the wide-ranging species *C. variegatus*. The three species co-occur in all habitats within the island's shallow saline lakes containing only two other fish species. Although generalist populations are ubiquitous across similar environments in the Caribbean with identical fish communities, the San Salvador clade is one of only two sympatric radiations of *Cyprinodon* and exhibits morphological diversification rates up to 51 times faster than those of other young clades for functional trophic traits (19).

We tested the effects of competition in the wild on the topography of fitness landscapes during *Cyprinodon* adaptive radiation by measuring the fitness of 1865 hybrids placed in high- and low-density field enclosures. Wild-caught breeding colonies of all three species were used to generate outbred F_2 hybrid populations for this experiment from F_1 hybrid intercrosses and backcrosses (20). Hybrid populations from two isolated lakes, Crescent Pond (CP) and Little Lake (LL), were generated independently. First, these laboratory-reared juvenile F_2 hybrids were measured for 16 morphological traits and implanted with coded wire tags. Next, we transported the hybrids to San Salvador Island and introduced them into a low- or high-density field enclosure in the respective lake from which their grandparents originated (CP: high/low-density

$n = 796/96$ hybrids; LL: $n = 875/98$ hybrids). After 3 months, we recovered all surviving hybrids and assigned a fitness of 1 relative to 0 for unrecovered hybrids. Fitness was also estimated from the growth (the increase in standard length) of survivors in enclosures.

To provide a biologically relevant frame of reference for hybrid morphology, we laboratory-reared and measured a purebred F_1 generation of all three species from each lake. We then plotted the hybrids in a discriminant morphospace separating the F_1 purebred species in each lake and visualized fitness landscapes for survival (Fig. 1) and growth (fig. S1). Morphospace coverage was reduced in LL, probably due to missing a backcross to the durophage.

Fitness landscape topography was complex but largely congruent between high-density enclosures in each lake for both survival and growth (Fig. 1 and fig. S1). A local fitness peak in the high-density CP enclosure, isolated by declining fitness in all directions, corresponded to the phenotype of the generalist species (Fig. 1, A, C, and E). Phenotypic similarity between hybrids on this local fitness peak and the laboratory-reared generalist species cannot be attributed to familial relatedness or shared environments (20). Instead, this correspondence demonstrates strong stabilizing selection on hybrid phenotypes closely resembling generalists. There was a similar trend in LL (Table 1 and Fig. 2). Survival also declined with increasing total phenotypic distance from the generalist in both high-density enclosures (fig. S2).

A second region of increased fitness [linear discriminant axis 1 (LD1) < 0 and LD2 < 0 in Fig. 1A: $n = 120$ hybrids, logistic z -score = -2.33 , $P = 0.020$] corresponded to the phenotype and the diet (inferred from $\delta^{15}\text{N}$ stable isotopes) of the hard-shelled-prey specialist (durophage; Figs. 1 and 2, figs. S3 and S4, and table S1). In the CP high-density enclosure, increased survival in this region was supported by parametric and permutation tests (20), although less strongly than the generalist peak. This fitness region was also significantly higher (permutation test, $n = 796$ hybrids, $P = 0.044$) than the generalist peak (Fig. 1E) and was robust to alternative calculations of the discriminant morphospace (figs. S5 and S6). A similar trend of increased survival of hybrids resembling the durophage specialist was observed in LL (Figs. 1 and 2 and Table 1). Furthermore, CP hybrid survivors in this region occupied a higher trophic position than those on the generalist peak ($\delta^{15}\text{N}$ stable isotope ratios: $n = 64$ hybrids, permutation test, $P = 0.048$; fig. S3 and table S1), reflecting the relative trophic positions of wild-caught durophage and generalist pupfishes ($F_{1,22} = 8.78$, $P = 0.007$; table S1). Thus, trophic divergence between hybrids mirrored trophic divergence in wild-caught species.

Hybrids resembling generalist and durophage phenotypes were separated by a valley of reduced fitness in both lakes (Fig. 2). Transects between species indicated significant disruptive

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Fig. 1. Survival fitness landscape for F₂ hybrids within the high-density field enclosure in each lake (left column: CP; right column: LL). **(A and B)** Individual F₂ hybrid survivors (black dots) and deaths (gray dots) plotted within the discriminant morphospace (LD1 and LD2) estimated from laboratory-reared purebred species. Heat colors indicate survival probabilities estimated from thin-plate splines fit to the data by generalized cross-validation (effective df: CP, 7.6; LL, 19.6). **(C and D)** Laboratory-reared purebred species are superimposed within 95% confidence ellipses (dashed lines: generalist, blue dots; durophage, green dots; scale-eater, red dots) for reference. **(E and F)** Relative heights of the fitness landscape.

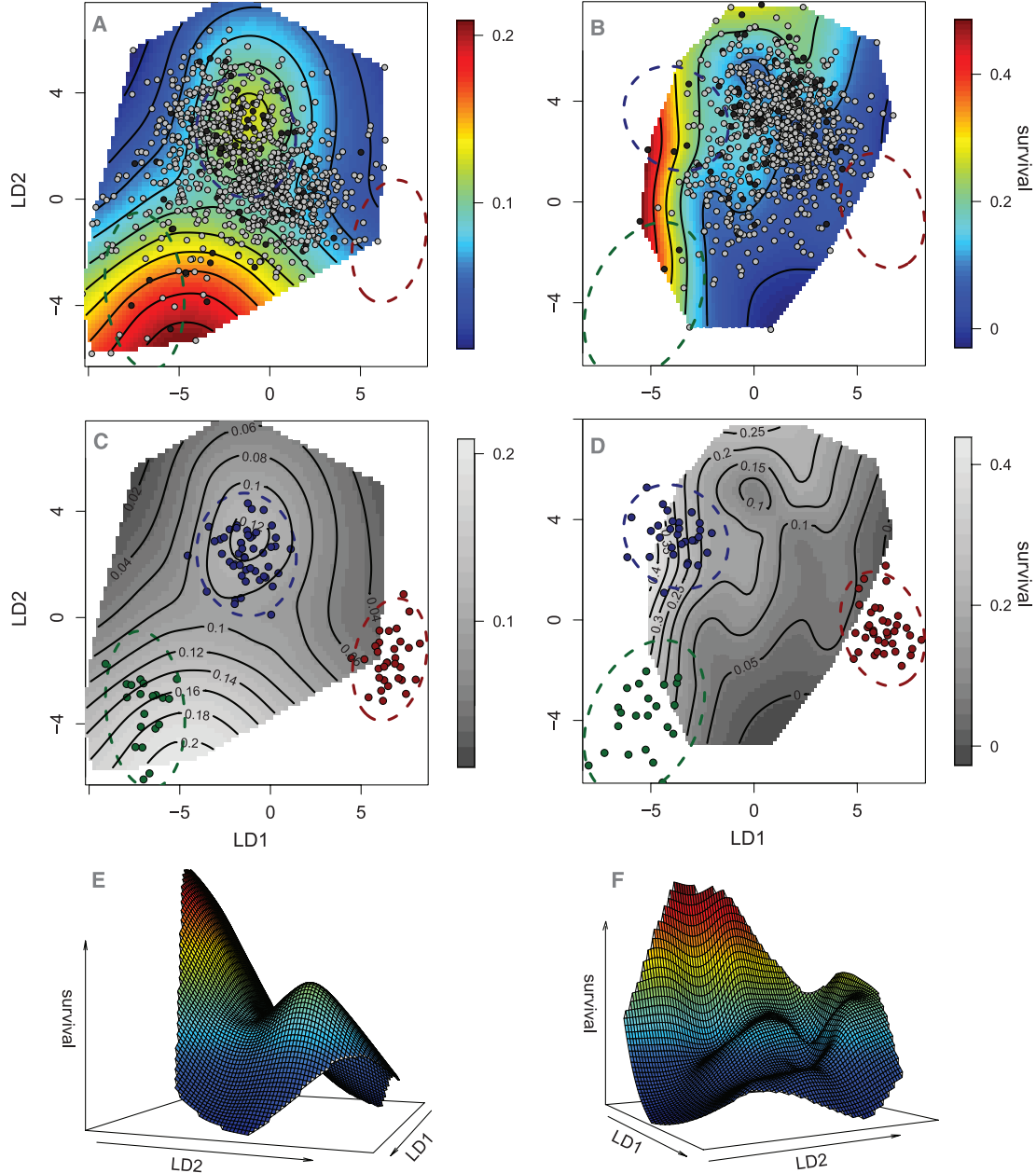


Table 1. Significance of local regions of stabilizing ($\gamma < 0$) and disruptive ($\gamma > 0$) selection within survival fitness landscapes for F₂ hybrids in high-density field enclosures (Fig. 1). The form of selection on hybrid phenotypes was tested for the quadratic intervals depicted in Fig. 2. Sample sizes within each interval are reported for each test. *n*, number of hybrids; GLM, generalized linear model.

Hybrid transect	Lake	Quadratic interval	γ	Survival		Growth		Joint†
				<i>n</i>	Logistic <i>P</i>	<i>n</i>	GLM <i>P</i>	Permutation <i>P</i>
Durophage versus generalist	CP	Fig. 2A (i)	−0.120	355	0.024*	46	0.963‡	0.006**
	LL	Fig. 2B (i)	−0.150	52	0.203	9	0.644	0.047*
	Combined							1.4×10^{-4} ***
	CP	Fig. 2A (ii)	0.160	359	0.005**	52	0.050*	0.002**
	LL	Fig. 2B (ii)	0.432	27	0.030*	8	0.911	0.043*
Generalist versus scale-eater	Combined							3×10^{-5} ****
	CP	Fig. 2C	−0.132	555	0.006**	64	0.734‡	0.001**
	LL	Fig. 2D	0.104	406	0.152	44	0.497‡	0.473

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. †Joint significance of selection gradients (γ) for both growth and survival (20). ‡Survival and growth did not agree on the form of selection (opposite signs), and the joint test used only survival data. Selection gradients (γ) for survival are shown.

selection on intermediate phenotypes (Table 1, Fig. 2). The form of selection on growth was not always consistent with survival (Table 1); however, multiple regions of increased fitness, corresponding to generalist and durophage phenotypes, were observed in both survival and growth fitness landscapes in the high-density CP enclosure and showed a similar trend in LL (Fig. 1 and fig. S1).

Hybrids resembling the scale-eater phenotype had greatly reduced survival and growth in both lakes at high and low densities (Figs. 1 and 2, fig. S1, and Table 1), demonstrating that low fitness over a large region of morphospace reproductively isolates this species from the others. Hybrid survivors resembling scale-eaters rarely ingested any scales (4 out of 11 relative to 49 out of 53 wild-caught scale-eaters);

thus, scale-eating may require an extreme phenotype, not recovered in hybrids, for successful performance. Alternatively, field enclosures may not support the scale-eating niche; however, we consider this unlikely because the frequency of hybrids resembling scale-eaters within high-density enclosures (CP, 0.6%; LL, 0.9%) was similar to wild scale-eater frequencies [1.4% (20)], prey density was higher in enclosures, and fitness did not vary between density treatments. Overall, the reduced fitness of intermediate and transgressive hybrids supports the importance of postzygotic extrinsic reproductive isolation.

High-density enclosures provided a more competitive environment than low-density enclosures: Survival was 4 to 7 times higher and growth was 1.4 to 2 times higher in low-density enclosures (mean survival: CP, 11.4% versus 71.9%; LL, 11.1% versus 43.9%; logistic $z = 11.8$, $P < 10^{-16}$; mean growth: CP, 0.196 versus 0.428 cm; LL, 0.164 versus 0.223 cm; $t = -2.8$, $P = 0.005$). The curvature of the fitness landscape was significantly greater in high-density enclosures in both lakes (Table 2 and figs. S1 and S7), supporting competition as the driver of multiple fitness peaks on the adaptive landscape. If intrinsic differences in fitness among hybrid phenotypes were responsible, complex fitness landscapes should occur in both field and laboratory environments. However, laboratory survival surfaces, estimated from hybrids that died during laboratory rearing, were flat (table S2 and fig. S8). Combined, these data indicate that multiple high-fitness regions were caused by competition for diverse resources, not intrinsic survival differences.

This complex fitness landscape paints an intriguing picture of niche diversification driven by competition in *Cyprinodon*. The generalist species sits atop a local fitness maximum separated by a valley from a higher-fitness region corresponding to specialization on hard-shelled prey. Stabilizing selection on generalist phenotypes could explain the rarity of trophic specialists within *Cyprinodon* despite their higher fitness: Sympatric adaptive radiations of trophic specialists may have evolved in only two places throughout the Caribbean because most generalist populations are stranded on an isolated local maximum. When subpopulations escape this generalist peak, perhaps through increased competition, ecological opportunity, and large effective population size, the higher fitness of trophic specialists drives a burst of diversification.

The early burst model of adaptive radiation predicts a fitness landscape with multiple peaks at the onset of adaptive radiation (1, 2, 4, 7, 8). We simulated phenotypic diversity within the ancestral population that gave rise to an adaptive radiation of pupfishes in order to measure the initial topography of the fitness landscape. In contrast to theory and examples demonstrating that high-frequency phenotypes cause a fitness minimum due to negative frequency-dependent selection (9, 10), some of the most frequent phenotypes in our field enclosures occurred on a local

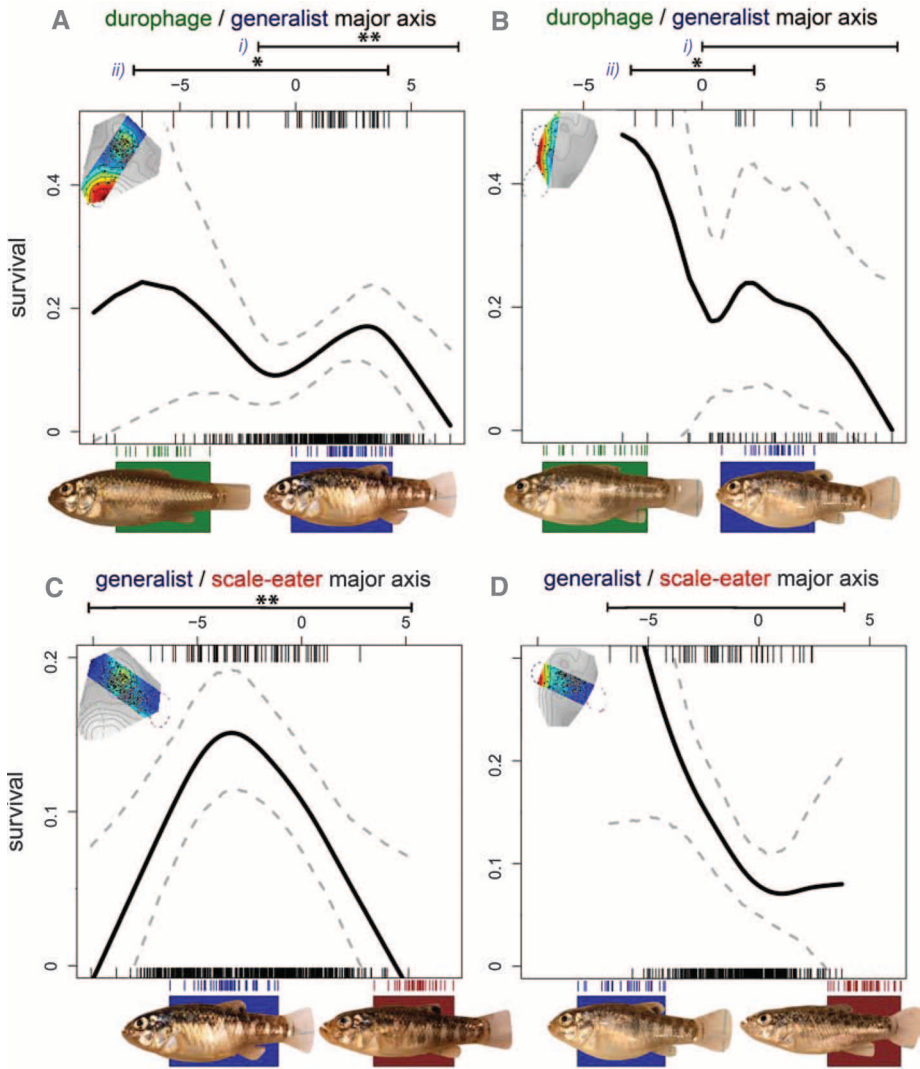


Fig. 2. Probability of F_2 hybrid survival in high-density field enclosures within local transects between species (left column, CP; right column, LL). Smoothing splines (black line) and 95% confidence intervals (dotted gray lines) indicate hybrid survival along major phenotypic axes between species ellipses from Fig. 1 (insets at upper left). (A and B) Major axis between hybrids resembling durophage or generalist species. (C and D) Major axis between hybrids resembling generalist or scale-eater species. Interior rug plots show F_2 hybrid survivors (upper) and deaths (lower). Exterior rug plots show laboratory-reared F_1 purebred species (generalist, blue; durophage, green; scale-eater, red) for reference. Quadratic intervals used for parametric analyses (Table 1) are indicated with significance from logistic regression.

Table 2. Effect of competition on fitness landscape curvature. Three permutation tests assessed the significance of greater survival surface curvature in high-density enclosures relative to low-density enclosures (fig. S7), controlling for different sample sizes and morphospace coverage. Significance was determined from the number of randomized samples equal to or greater than the observed difference between treatments (20). Effective degrees of freedom (EDF) indicate the smoothness of the surface estimated by generalized cross-validation.

Lake	Density	<i>n</i>	Thin-plate spline		Projection pursuit regression		Canonical rotation of γ	
			EDF	<i>P</i>	EDF	<i>P</i>	<i>F</i>	<i>P</i>
Crescent Pond	High	796	7.6	0.071*	3.1	0.065*	3.41	0.018*
	Low	96	3.0		2.0		0.01	
Little Lake	High	875	19.6	0.008**	6.7	0.006**	1.03	0.711
	Low	98	3.0		2.4		1.73	

* $P < 0.10$, * $P < 0.05$, ** $P < 0.01$.

fitness maximum, suggesting that the broader topography of adaptive landscapes is more strongly determined by stable performance constraints than frequency-dependent dynamics.

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Supplementary Materials

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Suppression of Oxidative Stress by β -Hydroxybutyrate, an Endogenous Histone Deacetylase Inhibitor

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Concentrations of acetyl-coenzyme A and nicotinamide adenine dinucleotide (NAD⁺) affect histone acetylation and thereby couple cellular metabolic status and transcriptional regulation. We report that the ketone body D- β -hydroxybutyrate (β OHB) is an endogenous and specific inhibitor of class I histone deacetylases (HDACs). Administration of exogenous β OHB, or fasting or calorie restriction, two conditions associated with increased β OHB abundance, all increased global histone acetylation in mouse tissues. Inhibition of HDAC by β OHB was correlated with global changes in transcription, including that of the genes encoding oxidative stress resistance factors FOXO3A and MT2. Treatment of cells with β OHB increased histone acetylation at the *Foxo3a* and *Mt2* promoters, and both genes were activated by selective depletion of HDAC1 and HDAC2. Consistent with increased FOXO3A and MT2 activity, treatment of mice with β OHB conferred substantial protection against oxidative stress.

Cellular metabolites such as acetyl-coenzyme A (acetyl-CoA) and nicotinamide adenine dinucleotide (NAD⁺) influence gene expression by serving as cofactors for epigenetic modifiers that mediate posttranslational modification of histones (1). The activity of histone acetyltransferases (HATs) is dependent on nuclear acetyl-CoA concentrations (2, 3) and the deacetylase activity of class III HDACs, also called sirtuins, is dependent on NAD⁺ concentrations (4). Class I (HDAC1, 2, 3, 8), class II (HDAC4, 5, 6, 7, 9, 10), and class IV (HDAC11) HDACs are zinc-dependent enzymes, but endogenous regulators are not known.

Small-molecule inhibitors of class I and class II HDACs include butyrate, a product of bacterial anaerobic fermentation (5). Butyrate is closely related to β -hydroxybutyrate (β OHB) (Fig. 1A), the major source of energy for mammals during prolonged exercise or starvation (6). Accumulation of β OHB in blood increases to 1 to 2 mM during fasting when the liver switches to fatty acid oxidation (7, 8), and to even higher concentrations during prolonged fasting (6 to 8 mM) (6) or in diabetic ketoacidosis (>25 mM) (9).

To determine whether β OHB might have HDAC inhibitor activity, we treated human embryonic kidney 293 (HEK293) cells with different amounts of β OHB for 8 hours, and measured

histone acetylation levels by Western blot with antibodies to acetylated histone H3 lysine 9 (AcH3_{K9}) and to acetylated histone H3 lysine 14 (AcH3_{K14}) (Fig. 1, B and C, and figs. S1 and S2). β OHB increased histone acetylation in a dose-dependent manner, even at 1 to 2 mM, which can occur in humans after a 2- to 3-day fast or strenuous exercise (6, 8, 10). Like butyrate, β OHB did not increase acetylation of α -tubulin, indicating that it inhibits class I HDACs but not the class IIb tubulin deacetylase, HDAC6.

To test the HDAC inhibitor activity of β OHB and its possible selectivity, we purified recombinant human HDACs after transient transfection of expression vectors for human epitope-tagged (FLAG) HDAC1, HDAC3, HDAC4, and HDAC6 in HEK293T cells. We purified the HDACs, incubated them with ³H-labeled acetylated histone H4 peptides, and measured their deacetylase activity (Fig. 1D) (11). β OHB inhibited HDAC1, HDAC3, and HDAC4 in a dose-dependent manner with a median inhibitory concentration (IC₅₀) of 5.3, 2.4, and 4.5 mM, respectively. The HDAC6 IC₅₀ was much higher (48.5 mM) (Fig. 1E), and β OHB did not inhibit HDAC6 activity on its natural substrate tubulin (fig. S3). To examine the possibility that histone acetylation was enhanced by increased concentration of acetyl-CoA (because β OHB is catabolized into acetyl-CoA in target tissues), we directly measured abundance of acetyl-CoA in β OHB-treated HEK293 cells, but no change was observed (fig. S4). We also tested a possible activating effect of β OHB on histone acetyltransferase activity of p300 and PCAF (P300/CBP-associated factor) but did not detect any change induced by β OHB (fig. S5). Thus, millimolar concentrations of β OHB appear to increase histone acetylation directly through HDAC inhibition. High concentrations of acetoacetate (AcAc) also inhibit class I and class IIa HDACs in vitro (fig. S6A) and in HEK293 cells (fig. S6B). However, the concentration of AcAc in blood is one-third or less than that of β OHB during fasting and therefore less likely to reach concentrations that would inhibit HDACs under physiological conditions (12). To test the relative contribution of β OHB,

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acetoacetate, and acetyl-CoA to histone acetylation in response to β OHB treatment, we depleted cells of β OHB dehydrogenases (BDH1,

2) with small interfering RNA. Both enzymes catalyze the transformation of β OHB into aceteto-

acetate, and their suppression had no effect on histone acetylation in response to β OHB up to 3mM. At higher concentrations of β OHB (10 and 30 mM), however, further increase in histone acetylation was suppressed by depletion of β OHB dehydrogenases (fig. S7, A and B), indicating that either AcAc or acetyl-CoA might also contribute to histone acetylation in cells exposed to concentrations of β OHB above the IC_{50} for HDACs.

To determine whether changes in β OHB concentrations in vivo might affect histone acetylation, we measured β OHB concentration in mouse serum after a 24-hour fast or in mice on calorie restriction (CR). We also used implanted osmotic pumps to administer exogenous β OHB or phosphate-buffered saline (PBS). β OHB concentrations increased to 1.5 ± 0.1 mM after a 24-hour fast, 0.6 ± 0.1 mM in mice on CR and 1.2 ± 0.1 mM with administration of β OHB via an intraperitoneal pump (Fig. 2A). We collected tissues from fed or 24-hour-fasted mice and measured histone acetylation by immunoblotting. Acetylation of H3_{K9} and H3_{K14} reflect the competing activities of HATs and HDACs and influence gene expression in several species, including humans (13). Acetylation of H3_{K9} and H3_{K14} increased significantly in several organs in fasted mice, particularly kidney (Fig. 2B and figs. S8 and S9). In kidney, histone acetylation (H3_{K9} and H3_{K14}) also increased two- to fivefold in mice under CR. Kidney histone acetylation and serum β OHB concentrations were strongly correlated for both histone H3_{K9} ($R^2 = 0.772$) and histone H3_{K14} ($R^2 = 0.863$) (Fig. 2C). We first focused on kidney, the organ with the largest changes in histone acetylation, to investigate the effects

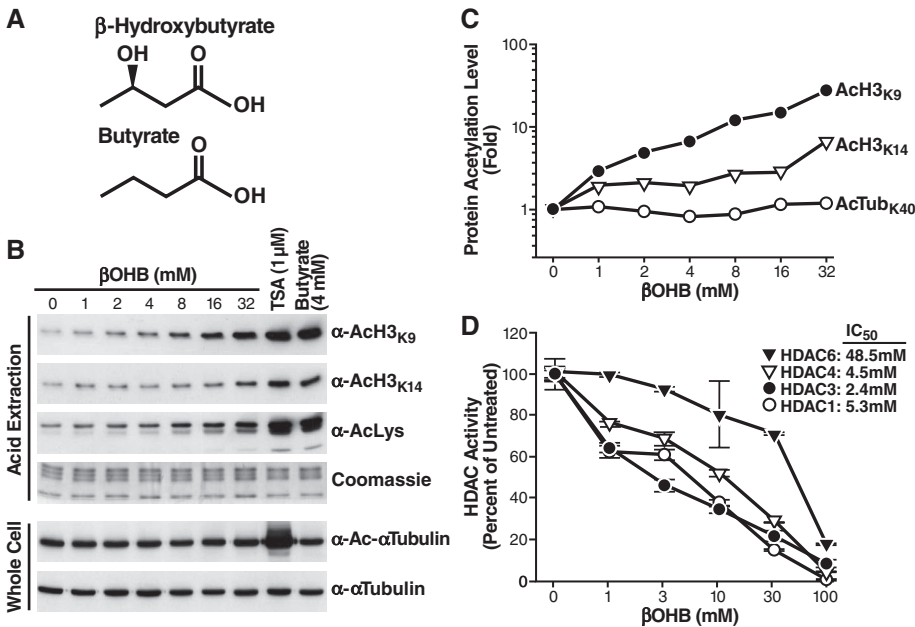


Fig. 1. Inhibition of HDACs by β OHB in vitro and in vivo. (A) Structures of β -hydroxybutyrate and butyrate. (B) Effect of β OHB, TSA, or butyrate on acetylation of histone H3 and tubulin. HEK293 cells were treated with the indicated concentrations of drugs for 8 hours. Histones were acid-extracted, and their acetylation was assessed by protein immunoblotting with anti-AcH3_{K9}, anti-AcH3_{K14}, or anti-acetyllysine (AcLys). Proteins from whole-cell extracts were analyzed by immunoblotting with antibodies to α -tubulin or Ac- α -Tubulin. (C) Quantification of acetylation levels from blots in (B), shown relative to untreated cells (β OHB 0 mM). (D) Inhibition of immunopurified HDACs by β OHB in vitro. Flag-tagged HDACs were expressed in HEK293 cells, immunoprecipitated, and incubated in vitro with a 3 H-labeled acetylated histone H4 peptide and the indicated concentrations of β OHB. HDAC activity is relative to the activity of each enzyme without β OHB. The IC_{50} values of β OHB are shown.

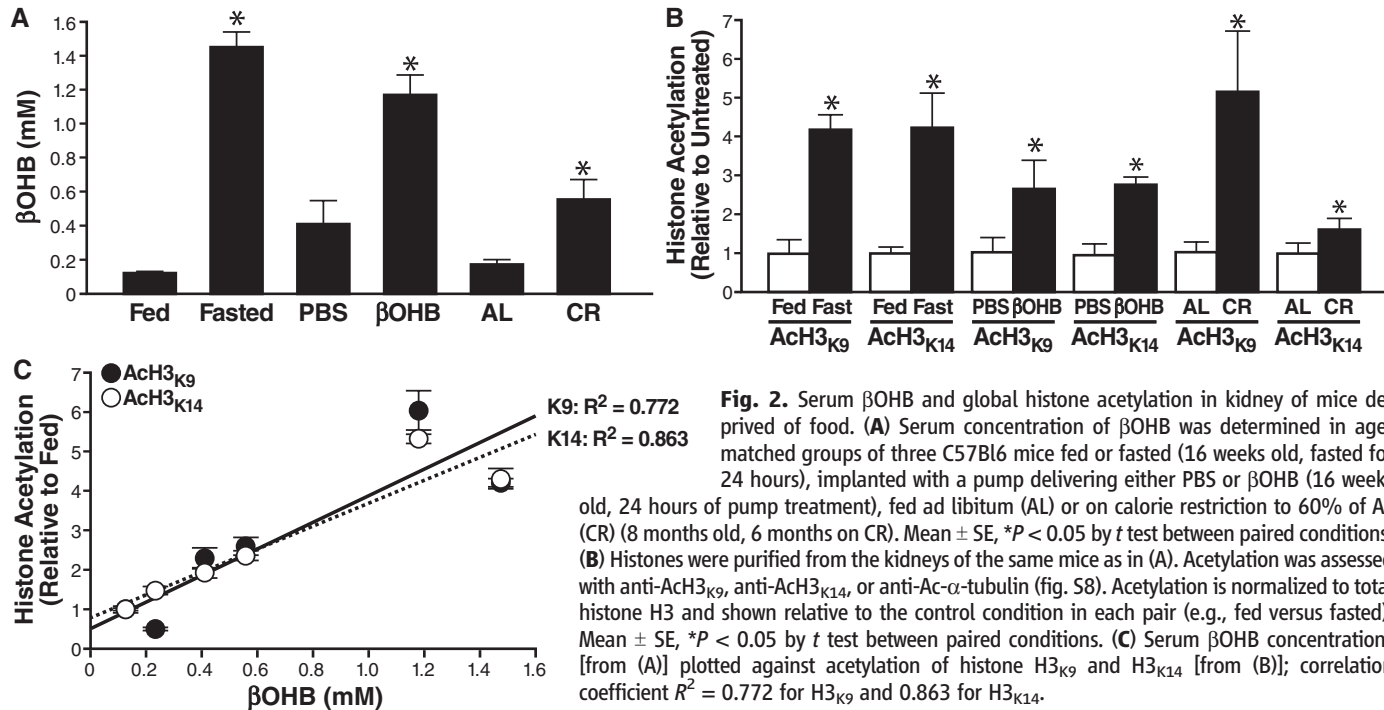


Fig. 2. Serum β OHB and global histone acetylation in kidney of mice deprived of food. (A) Serum concentration of β OHB was determined in age-matched groups of three C57Bl6 mice fed or fasted (16 weeks old, fasted for 24 hours), implanted with a pump delivering either PBS or β OHB (16 weeks old, 24 hours of pump treatment), fed ad libitum (AL) or on calorie restriction to 60% of AL (CR) (8 months old, 6 months on CR). Mean $\pm$ SE, * P < 0.05 by t test between paired conditions. (B) Histones were purified from the kidneys of the same mice as in (A). Acetylation was assessed with anti-AcH3_{K9}, anti-AcH3_{K14}, or anti-Ac- α -tubulin (fig. S8). Acetylation is normalized to total histone H3 and shown relative to the control condition in each pair (e.g., fed versus fasted). Mean $\pm$ SE, * P < 0.05 by t test between paired conditions. (C) Serum β OHB concentrations [from (A)] plotted against acetylation of histone H3_{K9} and H3_{K14} [from (B)]; correlation coefficient $R^2 = 0.772$ for H3_{K9} and 0.863 for H3_{K14}.

of β OHB on gene expression and cellular phenotype.

Histone acetylation induced by HDAC inhibitors is associated with transcriptional activation and repression of a subset of cellular genes (14). To identify genes whose expression changed in response to β OHB, we extracted mRNA for microarray analysis from mouse kidneys treated with β OHB or PBS for 24 hours. As β OHB is more abundant in fasting conditions, gene expression changes induced by β OHB may be a subset of those induced by fasting. Of 35,556 genes tested, 284 increased transcription in response to fasting (false discovery rate <0.2 , table S1). Four of the five genes with the largest changes in expression in response to β OHB were also activated in response to fasting as determined both by microarray and quantitative real-time polymerase chain reaction (QPCR) ($P < 0.001$ for such overlap via binomial distribution, tables S2 and S3). Ingenuity pathway analysis identified two of the five β OHB-induced genes (*Mt2* and *Lcn2*) as regulated by FOXO3A. Overall, we found five genes in the FOXO3A network (*Foxo3a*, *Mt2*, *Lcn2*, *Lem3*, and *Hbp1*) that had increased transcription in response to β OHB via QPCR (fig. S10). *Foxo3a*, a transcription factor, induces cell-cycle arrest and resistance to oxidative stress (15). Metallothionein

2 (*Mt2*), the gene with the greatest transcriptional response to β OHB treatment as determined by QPCR, also protects against oxidative stress (16). *Foxo3a* and *Mt2* mRNA expression is also modestly increased during CR as determined by QPCR (Fig. 3, A and B). Chromatin immunoprecipitation (ChIP) analysis of the *Foxo3a* and *Mt2* promoters with two distinct primer pairs for each promoter revealed increased histone H3_{K9} acetylation at both promoters after treatment of HEK293 cells with a high dose of β OHB (10 mM) (Fig. 3C).

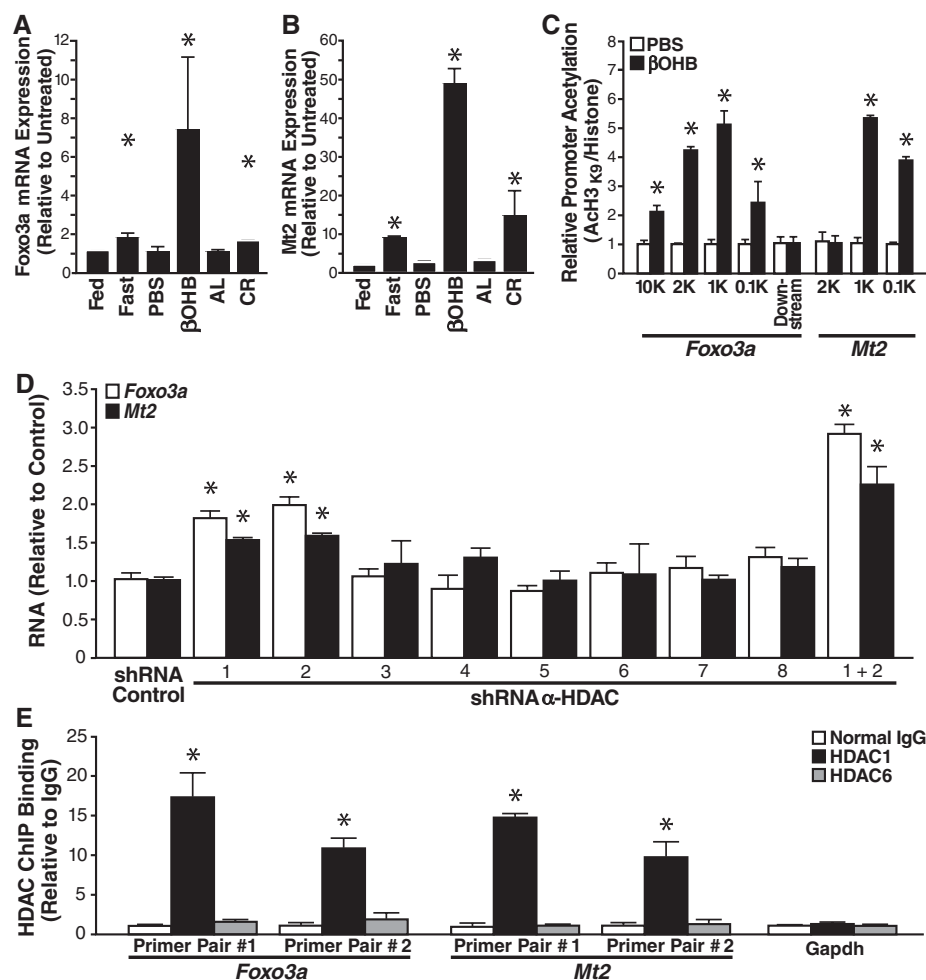
Next, we depleted cells of each class I and II HDAC with selective short hairpin-mediated RNAs (shRNAs). The shRNAs selective for each HDAC suppressed expression of their cognate HDAC by at least 60% (fig. S11). Depletion of HDAC1 or HDAC2 caused up-regulation of *Foxo3a* and *Mt2* mRNA by 1.8-fold and 1.5-fold, respectively (Fig. 3D). Depletion of both HDAC1 and 2 further increased accumulation of *Foxo3a* and *Mt2* mRNA (Fig. 3D). ChIP analysis revealed that HDAC1, but not HDAC6, was recruited to the *Foxo3a* and *Mt2* promoters and not recruited to the *Gapdh* promoter (Fig. 3E). Binding of HDAC1 to the *Foxo3a* promoter was unchanged by β OHB treatment; thus, HDAC catalytic activity appears not to be necessary for

promoter binding of HDAC1 (fig. S12). β OHB appears to induce local histone acetylation at the promoter of oxidative stress resistance genes, *Foxo3a* and *Mt2*, by inhibiting activity of HDACs 1 and 2.

Mitochondrial superoxide dismutase (Mn-SOD) and catalase are two other well-defined FOXO3A targets that contribute to its protective activity against oxidative stress (15, 17). Protein immunoblotting of kidney tissue isolated from PBS- or β OHB-treated mice showed increased expression of FOXO3A, Mn-SOD, and catalase (Fig. 4A and figs. S13 and S14).

The effect on expression of MT2, FOXO3A, MnSOD, and catalase indicated that β OHB might have protective activity against oxidative stress. Carbonyl derivatives are formed by a direct metal-catalyzed oxidative attack on the amino acid side chains of proline, arginine, lysine, and threonine. Carbonylation is irreversible and unrepairable and accumulates as organisms age (18). To test the possible protective role of β OHB against oxidative stress, we implanted mice with a subcutaneous pump delivering either β OHB or PBS for 24 hours. They then received an intravenous injection of paraquat, which induces accumulation of reactive oxygen species. Kidney tissue was isolated after

Fig. 3. Increased expression of oxidative stress resistance genes in cells exposed to β OHB. (A) Expression of *Foxo3a* under various conditions (see Fig. 2 for details) measured by QPCR. *Foxo3a* expression is normalized to abundance of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Mean $\pm$ SE, $*P < 0.05$ by *t* test between paired conditions. (B) Expression of *Mt2*, measured as in (A). (C) Promoters of *Mt2* and *Foxo3a* are enriched for acetylated histone H3K9 after β OHB treatment. HEK293 cells were treated with 10 mM β OHB or PBS for 24 hours. Chromatin was immunoprecipitated with anti-H3 or anti-AcH3_{K9}, and the purified DNA was analyzed with primer pairs specific for the *Foxo3a* or *Mt2* promoters. Results are the ratios of AcH3_{K9} to total histone H3. Mean $\pm$ SE, $*P < 0.05$ by *t* test between β OHB and PBS conditions. (D) HDAC depletion increases *Foxo3a* and *Mt2* mRNAs abundance. HEK293 cells were transfected with shRNAs specific for each class I or class II HDAC, and mRNA abundance was measured by QPCR 72 hours after transfection. Mean $\pm$ SE, $*P < 0.05$ by *t* test versus control shRNA. (E) HDAC1, but not HDAC6, is enriched at the promoters of *Mt2* and *Foxo3a*. ChIP analysis of the *Foxo3a* and *Mt2* promoters (two primer pairs per promoter) and *Gapdh* (one primer pair) from HEK293 cells with control immunoglobulin G (IgG), anti-HDAC1, or anti-HDAC6. Relative promoter binding of each HDAC is normalized to input *Gapdh*. Mean $\pm$ SE, $*P < 0.05$ by *t* test versus IgG control.



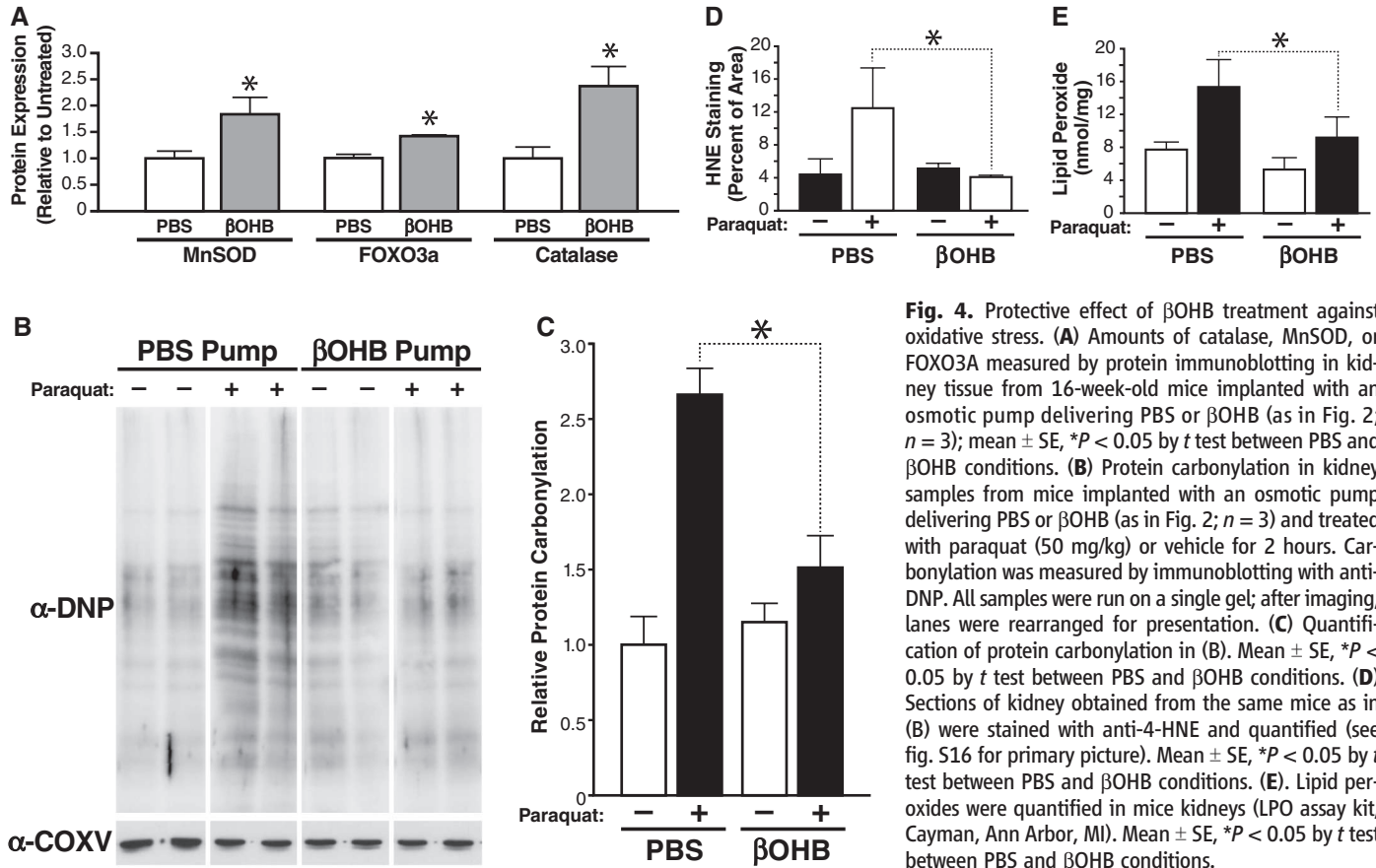


Fig. 4. Protective effect of β OHB treatment against oxidative stress. **(A)** Amounts of catalase, MnSOD, or FOXO3A measured by protein immunoblotting in kidney tissue from 16-week-old mice implanted with an osmotic pump delivering PBS or β OHB (as in Fig. 2; $n = 3$); mean $\pm$ SE, $*P < 0.05$ by t test between PBS and β OHB conditions. **(B)** Protein carbonylation in kidney samples from mice implanted with an osmotic pump delivering PBS or β OHB (as in Fig. 2; $n = 3$) and treated with paraquat (50 mg/kg) or vehicle for 2 hours. Carbonylation was measured by immunoblotting with anti-DNP. All samples were run on a single gel; after imaging, lanes were rearranged for presentation. **(C)** Quantification of protein carbonylation in **(B)**. Mean $\pm$ SE, $*P < 0.05$ by t test between PBS and β OHB conditions. **(D)** Sections of kidney obtained from the same mice as in **(B)** were stained with anti-4-HNE and quantified (see fig. S16 for primary picture). Mean $\pm$ SE, $*P < 0.05$ by t test between PBS and β OHB conditions. **(E)** Lipid peroxides were quantified in mice kidneys (LPO assay kit, Cayman, Ann Arbor, MI). Mean $\pm$ SE, $*P < 0.05$ by t test between PBS and β OHB conditions.

2 hours, and protein carbonylation was assayed by protein immunoblotting with an antibody to dinitrophenyl (DNP) after derivatization of protein with dinitrophenylhydrazine (DNPH) (Fig. 4B). Paraquat treatment of control mice receiving a PBS infusion led to a twofold increase in carbonylated proteins. This increase in protein carbonylation was significantly suppressed ($54 \pm 9\%$ decrease) in mice receiving β OHB (Fig. 4C).

We also examined another marker of oxidative stress: lipid peroxidation. 4-Hydroxynonenal (4-HNE) is a degradation product of polyunsaturated lipid and accumulates in response to oxidative stress (19). Kidney tissue sections from PBS- or paraquat-treated mice were stained with an antibody to 4-HNE, and the amount of 4-HNE staining was quantified with imaging software (fig. S15). Paraquat treatment increased 4-HNE staining threefold in control mice (PBS) (Fig. 4D). This increase was completely suppressed in mice treated with β OHB. Lipid peroxides were also directly quantified in an enzyme-linked immunosorbent assay that measures conversion of ferrous ions to ferric ions. A twofold increase in lipid peroxide in response to paraquat was suppressed significantly by β OHB treatment (Fig. 4E). Thus, β OHB protects against paraquat-induced oxidative stress in mouse kidney.

Our observation that β OHB is an endogenous HDAC inhibitor present in organisms at millimolar concentrations during prolonged

fasting and CR reveals an example of integration between metabolic status and epigenetic changes. We show that changes in histone acetylation and gene expression caused by β OHB promote stress resistance in the kidney. Future studies should investigate the specific gene expression and physiological effects of β OHB in other tissues. For example, low-carbohydrate diets that induce substantial ketogenesis are broadly neuroprotective and enhance resistance of neurons to oxidative damage (20). In addition, reduction in HDAC activity by either genetic manipulation or chemical inhibition extends life span in *Drosophila* (21, 22). Inhibition of HDACs by β OHB might contribute to the beneficial effect of ketogenic diets and may be one mechanism by which calorie restriction confers health benefits.

References and Notes

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Supplementary Materials

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Materials and Methods
Figs. S1 to S15
Tables S1 to S3
References (23–26)

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The COMPASS Subunit Spp1 Links Histone Methylation to Initiation of Meiotic Recombination

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During meiosis, combinatorial associations of genetic traits arise from homologous recombination between parental chromosomes. Histone H3 lysine 4 trimethylation marks meiotic recombination hotspots in yeast and mammals, but how this ubiquitous chromatin modification relates to the initiation of double-strand breaks (DSBs) dependent on Spo11 remains unknown. Here, we show that the tethering of a PHD-containing protein, Spp1 (a component of the COMPASS complex), to recombinationally cold regions is sufficient to induce DSB formation. Furthermore, we found that Spp1 physically interacts with Mer2, a key protein of the differentiated chromosomal axis required for DSB formation. Thus, by interacting with H3K4me3 and Mer2, Spp1 promotes recruitment of potential meiotic DSB sites to the chromosomal axis, allowing Spo11 cleavage at nearby nucleosome-depleted regions.

Meiotic recombination is initiated by the introduction of DNA double-strand breaks (DSBs) by Spo11, a meiosis-specific transesterase, which is highly conserved throughout evolution (1). To ensure at least one crossover per chromosome pair, a large number of DSBs are formed in each meiotic cell, and their occurrence is controlled at multiple levels, comprising both higher-order chromosome structure and local determinants (2). In *Saccharomyces cerevisiae*, ~150 DSBs are formed per meiosis, preferentially in promoter regions and with a highly variable frequency. DSB sites are largely independent of DNA sequence composition (3). The control of DSB formation also depends on a number of Spo11-accessory proteins that have been shown to form subcomplexes, but whose functions are only partially understood (4). In particular, the Mer2/Mei4/Rec114 complex plays a role in linking replication to subsequent DSB formation through Mer2 phosphorylation (5–7). This complex is also thought to tether potential DSB sites located on chromatin loops to the highly differentiated meiotic chromosome axis, leading to stepwise activation of Spo11 cleavage (8–10).

In *S. cerevisiae*, all H3K4 methylation is carried out by the COMPASS protein complex that includes Set1, the catalytic subunit, which acts as a scaffold for the other structural and regulatory

components (11). In particular, the PHD-finger subunit Spp1 specifically regulates the H3K4me3 state (11, 12). Our previous studies revealed that the absence of Set1 severely reduces meiotic DSB

levels (13, 14) and that the level of H3K4me3 is constitutively higher near DSB sites (14). However, beyond these correlations, the mechanistic link between H3K4 methylation and DSB formation has remained elusive (15).

To uncover functional connections, we first asked whether the mutation of each COMPASS subunit affected DSB frequencies at natural recombination hotspots (16). Similar to the deletion of *SET1*, the absence of each COMPASS subunit, except Shg1, reduced DSB frequencies at the *BUD23* (Fig. 1A) and *CYS3* hotspots (fig. S1A; strain genotypes in table S1). We observed the frequency reduction for Spp1, which is specifically required for H3K4me3 formation. The similar effect of *set1Δ* and *spp1Δ* mutants on DSB formation at hotspots also extends to DSB formation near the naturally cold *PES4* locus (fig. S1B) (14). To check if the decrease in DSB formation at hotspots reflected the role of only the COMPASS complex on H3K4 methylation, we asked whether this decrease was recapitulated by mutation of the H3K4 residue. At *BUD23*, we observed equivalent DSB reduction in the *set1Δ* and *H3K4R* strains (fig. S1C). Parallel analysis of the *set1Δ H3K4R* double mutant revealed an identical effect,

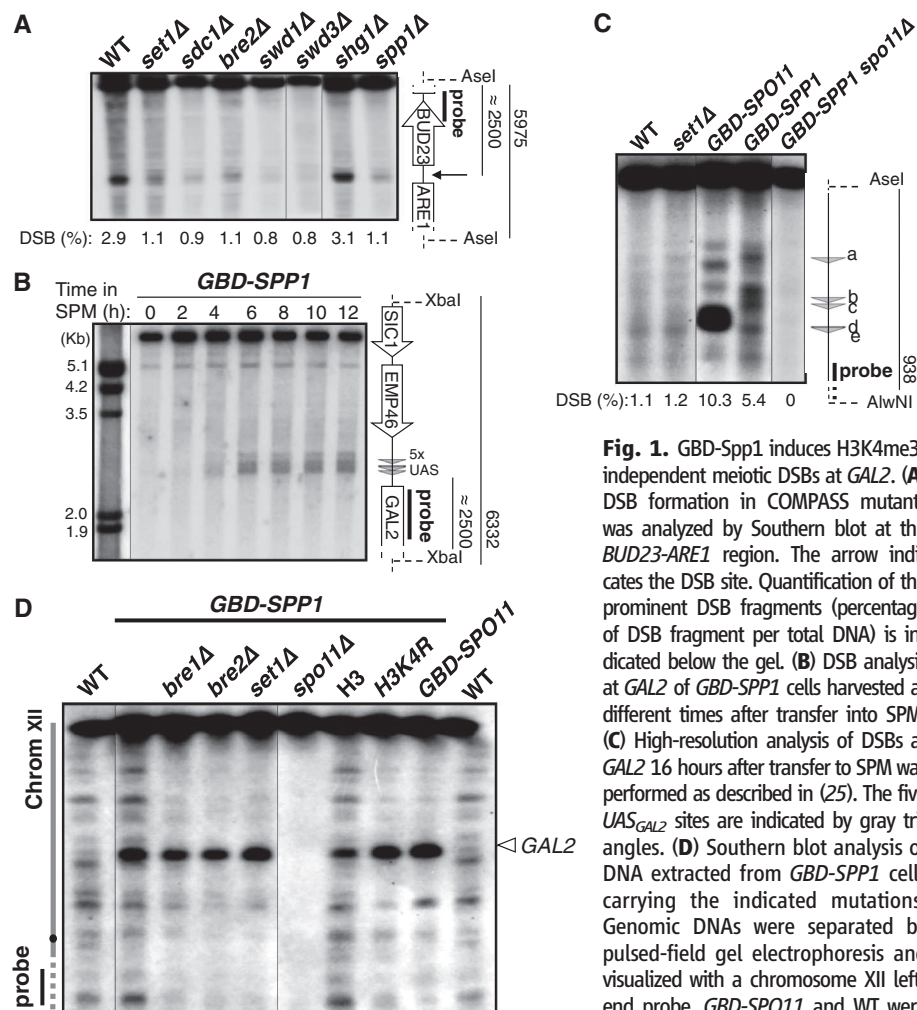


Fig. 1. GBD-Spp1 induces H3K4me3-independent meiotic DSBs at *GAL2*. (A) DSB formation in COMPASS mutants was analyzed by Southern blot at the *BUD23-ARE1* region. The arrow indicates the DSB site. Quantification of the prominent DSB fragments (percentage of DSB fragment per total DNA) is indicated below the gel. (B) DSB analysis at *GAL2* of *GBD-SPP1* cells harvested at different times after transfer into SPM. (C) High-resolution analysis of DSBs at *GAL2* 16 hours after transfer to SPM was performed as described in (25). The five *UAS_{GAL2}* sites are indicated by gray triangles. (D) Southern blot analysis of DNA extracted from *GBD-SPP1* cells carrying the indicated mutations. Genomic DNAs were separated by pulsed-field gel electrophoresis and visualized with a chromosome XII left-end probe. *GBD-SPO11* and WT were used as controls.

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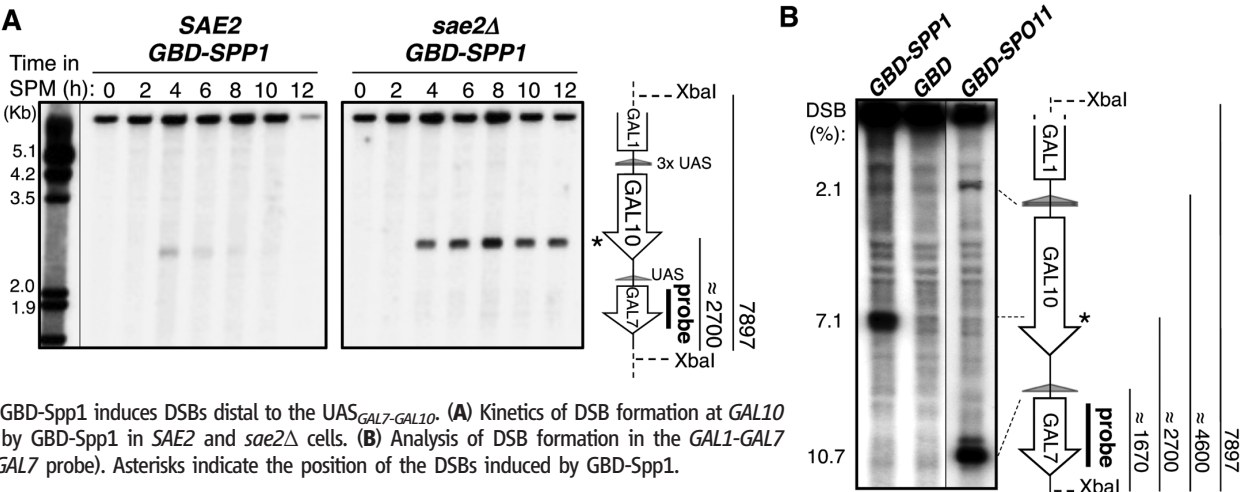


Fig. 2. GBD-Spp1 induces DSBs distal to the $UAS_{GAL7-GAL10}$. **(A)** Kinetics of DSB formation at *GAL10* induced by GBD-Spp1 in *SAE2* and *sae2Δ* cells. **(B)** Analysis of DSB formation in the *GAL1-GAL7* region (*GAL7* probe). Asterisks indicate the position of the DSBs induced by GBD-Spp1.

underscoring their epistatic relationship (fig. S1C). Together, these observations strongly support a role for the COMPASS complex and H3K4 trimethylation in the control of DSB formation and tie them to the same pathway.

To elucidate the role of H3K4me3, Set1, and Spp1 in DSB formation, we next asked whether the tethering of Set1 and Spp1 proteins to UAS_{GAL} (UAS, upstream activating sequence) binding sites located in cold DSB regions was sufficient to stimulate DSB formation, as previously observed for the fusion of the Gal4 binding domain (GBD) with Spo11 (17). We generated and expressed in-frame fusions of the coding region with the GBD. With respect to H3K4 methylation and DSB formation, both GBD-Set1 and GBD-Spp1 fusions behaved the same as the wild type, indicating no adverse interference with COMPASS and the Spo11 machinery (fig. S2 and table S2). We examined DSB formation in the naturally cold *GAL2* locus, which contains five UAS_{GAL} sequences in its promoter. Notably, the GBD-Set1 construct stimulated DSBs near the UAS_{GAL2} sites (fig. S3, A and B). In terms of genetic requirements, the data reported in fig. S3, C to E, establish that the GBD-Set1-induced DSBs were not associated with a coincident increase in H3K4me3 at *GAL2* but did depend on (i) the presence of Spo11, (ii) the integrity of the COMPASS, (iii) the histone methyl transferase activity of Set1, and (iv) the presence of the H3 lysine 4 residue.

Similar analyses revealed that GBD-Spp1 strongly stimulated DSB formation at *GAL2*. These DSBs appeared and disappeared upon repair with similar kinetics as natural DSBs (Fig. 1, B and C, see also Fig. 2A). Notably, DSBs targeted by GBD-Spp1 required neither the presence of Set1 or Bre2 nor the E3 ligase Bre1 that controls H3K4 trimethylation through the ubiquitinylation of H2B (18). In agreement with this observation, DSBs resulting from the tethering of Spp1 also appeared in the *H3K4R* mutant (Fig. 1D). Tethering Spp1 to the *GAL1/GAL10* and *GAL7* loci also efficiently induced DSBs (Fig. 2A), but, surprisingly, these breaks did not occur

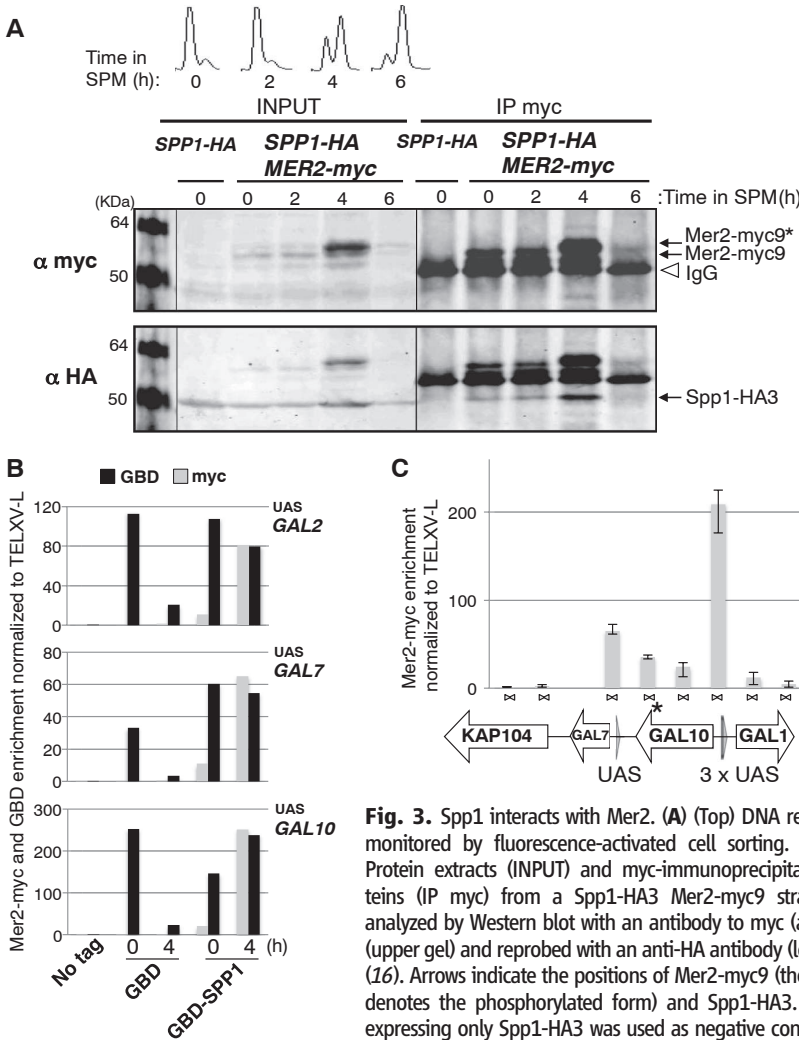
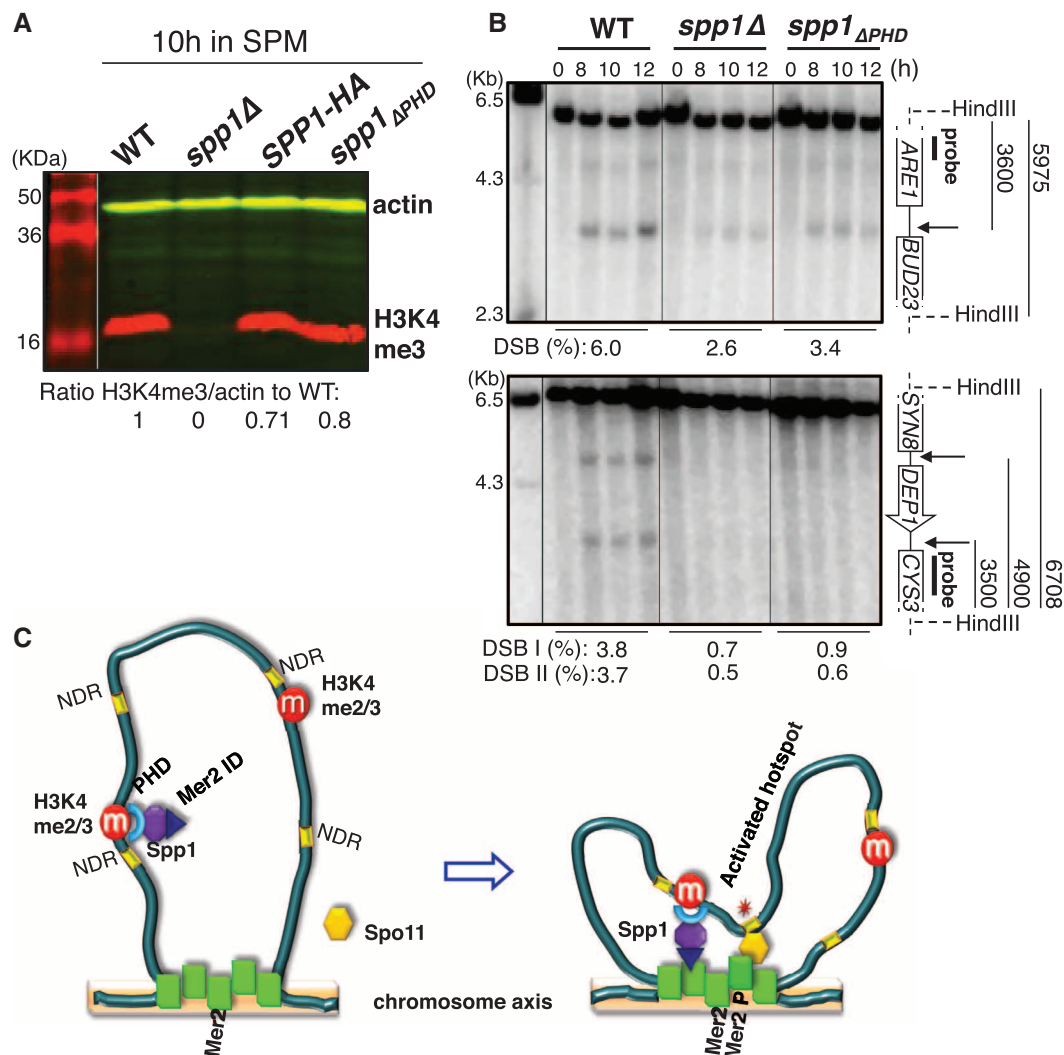


Fig. 3. Spp1 interacts with Mer2. **(A)** (Top) DNA replication monitored by fluorescence-activated cell sorting. (Bottom) Protein extracts (INPUT) and myc-immunoprecipitated proteins (IP myc) from a Spp1-HA3 Mer2-myc9 strain were analyzed by Western blot with an antibody to myc (anti-myc) (upper gel) and reprobred with an anti-HA antibody (lower gel) (16). Arrows indicate the positions of Mer2-myc9 (the asterisk denotes the phosphorylated form) and Spp1-HA3. A strain expressing only Spp1-HA3 was used as negative control. IgG, immunoglobulin G. **(B)** GBD, GBD-SET1, and GBD-SPP1 strains expressing Mer2-myc9 were harvested after 0 and 4 hours in SPM. Binding of the GBD-fusion proteins and Mer2-myc9 at the *GAL2*, *GAL7*, and *GAL10* UASs was analyzed by chromatin immunoprecipitation (ChIP) with anti-GBD and anti-myc antibodies. Polymerase chain reaction (PCR) primers specific for each GAL_{UAS} and TELXV-L (control) were used to amplify immunoprecipitated DNAs (16). **(C)** Chromatin binding of Mer2-myc9 in the GBD-SPP1 strain was analyzed by ChIP-quantitative PCR after 4 hours in SPM along the genomic region comprising *GAL7*, *GAL10*, and *GAL1* at positions indicated by two convergent arrowheads. The asterisk indicates the Gal1 ucut promoter. Error bars represent SDs from three independent experiments.

Fig. 4. The PHD-finger domain of Spp1 regulates meiotic DSB formation. **(A)** Analysis of H3K4me3 levels (16). **(B)** DSB formation at the *BUD23* and *CYS3* hotspots. **(C)** Extension of the chromatin loop-axis model. The tethering of a H3K4me-rich region to the chromosomal axis via the Spp1-Mer2 interaction allows Spo11 cleavage at a proximal nucleosome-depleted region (NDR). The red circles labeled with "m" indicate di- and trimethylated H3K4.



at the same location as those observed in the *GBD-SPO11* (Fig. 2B). Whereas tethering of Spo11 introduced DSBs in the vicinity the *UAS* sites, tethering of Spp1 to the same sites produced cleavage in the 3' end of the *GAL10* coding sequence (Fig. 2B and fig. S4A). As for the majority of meiotic DSBs in *S. cerevisiae*, occurring in promoter regions, the unexpected location of these GBD-Spp1-induced DSBs coincides with the promoter of the well-characterized antisense *GAL1* ucet (SUT013) noncoding RNA. This RNA is expressed in meiosis (19) under the control of the transcription factor Reb1 (20). This situation is also consistent with the high-resolution, genome-wide map of DSBs, which revealed a high enrichment of Spo11 binding in nucleosome-depleted regions adjacent to the Reb1 binding sites (3). As in *GAL2*, the DSBs generated at *GAL10* required Spo11 and occurred in the absence of H3K4 methylation (fig. S4B). Together, these results demonstrated that the tethering of Spp1 efficiently stimulated Spo11-dependent DSBs independently of H3K4 methylation.

A simple interpretation of the above results is that Spp1 recruits a component of the DSB

machinery. To search for interaction partners, we used the full-length Spp1 protein to perform yeast two-hybrid screening (16). We readily identified Set1 and Mer2 as prominent interactors of Spp1 (table S3). We mapped the Spp1-interacting region of Mer2 to the central part of the protein (residues 105 to 172) (fig. S5A). We validated the Mer2-Spp1 interaction by glutathione *S*-transferase-Mer2 pull-down experiments using various in vitro-translated Spp1 polypeptides. We found that the 131-amino acid C-terminal region of Spp1 was required for interaction with Mer2 (fig. S5B). Finally, we used tagged versions of both proteins to examine the in vivo interaction of Spp1 and Mer2 during meiosis. The C-terminally tagged *SPP1-HA3* strain showed a level of H3K4me3 near that of the wild type and a slight reduction (35%) of DSB formation at the *BUD23* hotspot, but, nevertheless, wild-type (WT) spore viability, indicating efficient DSB formation throughout the genome (fig. S6 and table S2). Spp1-HA3 was efficiently coimmunoprecipitated with Mer2-myc9 during meiosis, with a peak at 4 hours (Fig. 3A). Deoxyribonuclease and phosphatase treatments of the Myc immunoprecipitated pro-

teins from the double-tag strain did not reduce the recovery of Spp1-HA (fig. S5, B and C), suggesting an interaction of Spp1 with the nonphosphorylated form of Mer2 (5, 7, 9). A schematic representation of the Spp1-Set1 and Spp1-Mer2 interaction domains is illustrated in fig. S5D.

The essential role of Mer2 in DSB formation and its interaction with Spp1 prompted us to determine whether Mer2 was present at the Spp1-tethered DSB sites. At the control *CTR86* and *SED4* regions known to associate with Mer2 in WT cells (9), we found that Mer2 was enriched in the strains expressing GBD and GBD-Spp1 (fig. S7A), suggesting that the expression of these GBD fusion proteins did not alter chromatin occupancy of Mer2. Four hours after transfer to the sporulation media (SPM), Mer2 was strongly enriched at the *UAS_{GAL}* sites in the strain expressing GBD-Spp1, but not in the strains expressing GBD alone (Fig. 3B). Importantly, Mer2-myc was also enriched in the vicinity of the *Gall* ucet promoter, where the major DSB site is detected (Fig. 3C). This enrichment appears to be specific, as its level is greater than the one expected to spread for the *UAS* sites.

Examination of the genetic requirements for DSB formation indicated that the cleavage site induced by the tethering of Spp1 (i) was independent of Set1, (ii) required Mer2, and (iii) did not result from Spp1 overexpression (fig. S7B). The previously described Spp1 zinc finger-like domain (SZF) was included in the Mer2-interacting region (21). We deleted the SZF canonical CXXC motif in GBD-Spp1 (GBD-Spp1_{ΔCXXC}) and tested whether deleting this evolutionarily conserved motif impairs DSB formation and Mer2 binding at *GAL10* (16). DSB formation and Mer2 binding were both strongly reduced at *GAL10*, providing a strong correlation between the ability of GBD-Spp1 to recruit Mer2 at *GAL10* and DSB formation (fig. S8). This finding outlines the importance of this interaction for DSB formation.

What is the role of H3K4 methylation in the control of DSB formation? We found that the amount of Spp1 was strongly reduced both in the absence of Set1 and in COMPASS mutants affecting the stability of Set1, but either remained normal in the catalytically inactive *set1G951S* mutant or was slightly reduced in the *H3K4R*, *bre2Δ*, and *sdcl1Δ* mutants (fig. S9, A and B). Therefore, decreased levels of Spp1 might only partially explain the DSB formation defect of mutants that are compromised in H3K4 methylation. Our interpretation is that the function of Spp1 in DSB formation depends on H3K4 methylation through an interaction of its PHD-finger with H3K4me2/3 (21). We therefore deleted the PHD domain of Spp1 (16) and tested whether the presence of the PHD domain was important for H3K4me3 and DSB formation. We detected a level of H3K4me3 near that of the wild type in the *spp1_{ΔPHD}* mutant (Fig. 4A), whereas DSB formation was clearly reduced at the *BUD23* and *CYS3* hotspots (Fig. 4B). We conclude that the Spp1 PHD domain itself plays a specific role in DSB formation.

Our work led us to propose an enriched chromatin loop-axis model (Fig. 4C) for the regulation of DSB formation that addresses how the meiotic DSB sites are mechanistically selected. We propose that the interaction between the COMPASS subunit, Spp1, and Mer2 brings potential meiotic DSB sites to the chromosome axis for further downstream events that will ultimately lead to Spo11-dependent DSB formation at axis-proximal regions that are depleted of nucleosomes. In mammals, H3K4 methylation has been reported to be enriched at recombination hotspots where the meiosis-specific PRDM9 H3K4 methyltransferase is known to act (22–24). How PRDM9 connects to the mammalian DSB machinery is not known, but, as in yeast, it may be the consequence of a direct interaction with a protein of the DSB machinery. Our results indicate that H3K4me3 is required for the function of Spp1, probably through its recognition by the PHD domain within Spp1, and this requirement can be bypassed by tethering Spp1 to the DNA locus. The broadly localized H3K4me3 modifi-

cation has the virtue of permitting the initiation of recombination at numerous places of the genome, a molecular strategy that ensures a large diversity of recombinant haplotypes to be transmitted by the gametes. In conclusion, this model offers a clue of how chromosome structure and DSB regulation are interrelated, and it attributes a pivotal role to Spp1 in the recruitment of components acting at meiotic DSB sites to the chromosomal axis.

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Supplementary Materials

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Materials and Methods
Figs. S1 to S9
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JNK Expression by Macrophages Promotes Obesity-Induced Insulin Resistance and Inflammation

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The cJun NH₂-terminal kinase (JNK) signaling pathway contributes to inflammation and plays a key role in the metabolic response to obesity, including insulin resistance. Macrophages are implicated in this process. To test the role of JNK, we established mice with selective JNK deficiency in macrophages. We report that feeding a high-fat diet to control and JNK-deficient mice caused similar obesity, but only mice with JNK-deficient macrophages remained insulin-sensitive. The protection of mice with macrophage-specific JNK deficiency against insulin resistance was associated with reduced tissue infiltration by macrophages. Immunophenotyping demonstrated that JNK was required for pro-inflammatory macrophage polarization. These studies demonstrate that JNK in macrophages is required for the establishment of obesity-induced insulin resistance and inflammation.

Obesity is an important public health problem that is associated with inflammation, cardiovascular disease, metabolic syndrome, and type 2 diabetes (1). Tissue infiltration by macrophages is a major contributor to inflammation and insulin resistance (2). Tissue macrophages comprise multiple populations (3); however, there are two well-known subtypes that are capable of dynamic interconversion (4). Classically activated macrophages (M1) induced by interferon- γ (IFN- γ) or endotoxin promote interleukin-12 (IL12)-mediated T helper 1 (T_H1)

immune responses. Alternatively, activated macrophages induced by IL4 or IL13 (M2a), immune complexes (M2b), and the anti-inflammatory cytokines IL10 or transforming growth factor- β (M2c) can promote T_H2 immune responses and mediate wound healing, tissue repair, and the resolution of inflammation. Obesity increases tissue infiltration by macrophages (5) and polarization to the pro-inflammatory M1 state (fig. S1) (6, 7). Indeed, the inflammation associated with M1-polarized tissue macrophages is implicated in the development of obesity-related insulin resistance (8–11).

Signal transduction pathways in macrophages that are responsive to obesity represent possible targets for therapeutic intervention in obese patients. One example is the stress-responsive cJun NH₂-terminal kinase (JNK) signal transduction pathway that is activated by obesity and is required for obesity-induced insulin resistance (12). Two genes (*Jnk1* and *Jnk2*) encode JNK proteins in peripheral insulin-responsive tissues. JNK1 plays a critical role in adipose tissue and muscle (but not liver) during the development of insulin resistance (12). JNK1 in the hypothalamus is also required for high-fat diet (HFD)-induced obesity (12). Whether JNK plays a role in macrophages is

unclear, with evidence both for and against having been reported (13–15). For instance, JNK1-specific deficiency in macrophages (14) and JNK2-deficiency (16) causes no defect in obesity-induced insulin resistance, which suggests that JNK expression in macrophages may play no role. Alternatively, JNK1 and JNK2 may function redundantly. Studies of the effect of compound deficiency of JNK1 plus JNK2 in macrophages are required to distinguish between these possibilities.

To test the role of macrophage JNK, we established control (*Lyz2-Cre⁺Jnk1^{+/+}Jnk2^{+/+}*) mice and mice with a macrophage-specific ablation of JNK (*Lyz2-Cre⁺Jnk1^{LoxP}/LoxP⁺Jnk2^{LoxP}/LoxP⁺*) (fig. S2). Genotype analysis demonstrated specific disruption of the *Jnk1* and *Jnk2* genes in macrophages isolated from JNK-deficient (Φ^{KO}) mice, but not control (Φ^{WT}) mice (fig. S2). This conclusion was confirmed through immunoblot analysis of JNK expression in macrophages isolated from Φ^{WT} and Φ^{KO} mice (fig. S3A). Impaired phosphorylation of the canonical JNK substrate cJun (17) in Φ^{KO} macrophages confirmed that JNK signaling was disrupted in these cells (fig. S3, B and C).

Comparison of Φ^{WT} and Φ^{KO} mice demonstrated that these animals exhibited similar obesity when fed a HFD, although a slight reduction in lean mass was detected in Φ^{KO} mice as compared with that in Φ^{WT} mice fed a normal chow diet (fig. S4). Chow-fed Φ^{WT} and Φ^{KO} mice displayed similar insulin and glucose tolerance and blood concentrations of glucose and insulin (Fig. 1, A to E). Feeding a HFD to Φ^{WT} mice caused hyperglycemia, hyperinsulinemia, and intolerance to both glucose and insulin (Fig. 1, A to E). In contrast, Φ^{KO} mice were protected against these effects of a HFD. Specifically, macrophage-specific JNK-deficiency prevented HFD-induced hyperglycemia and hyperinsulinemia, and also improved both insulin and glucose tolerance as compared with that of HFD-fed Φ^{WT} mice (Fig. 1, A to E). In contrast, the blood concentration of glycerol and free fatty acids in HFD-fed Φ^{WT} and Φ^{KO} mice was similar (fig. S5), suggesting that macrophage-specific JNK deficiency may not prevent obesity-associated lipolysis. Together, these data demonstrate similar diet-induced obesity phenotypes of Φ^{WT} and Φ^{KO} mice; however, the HFD-fed Φ^{KO} mice remained insulin sensitive as compared with HFD-fed Φ^{WT} mice.

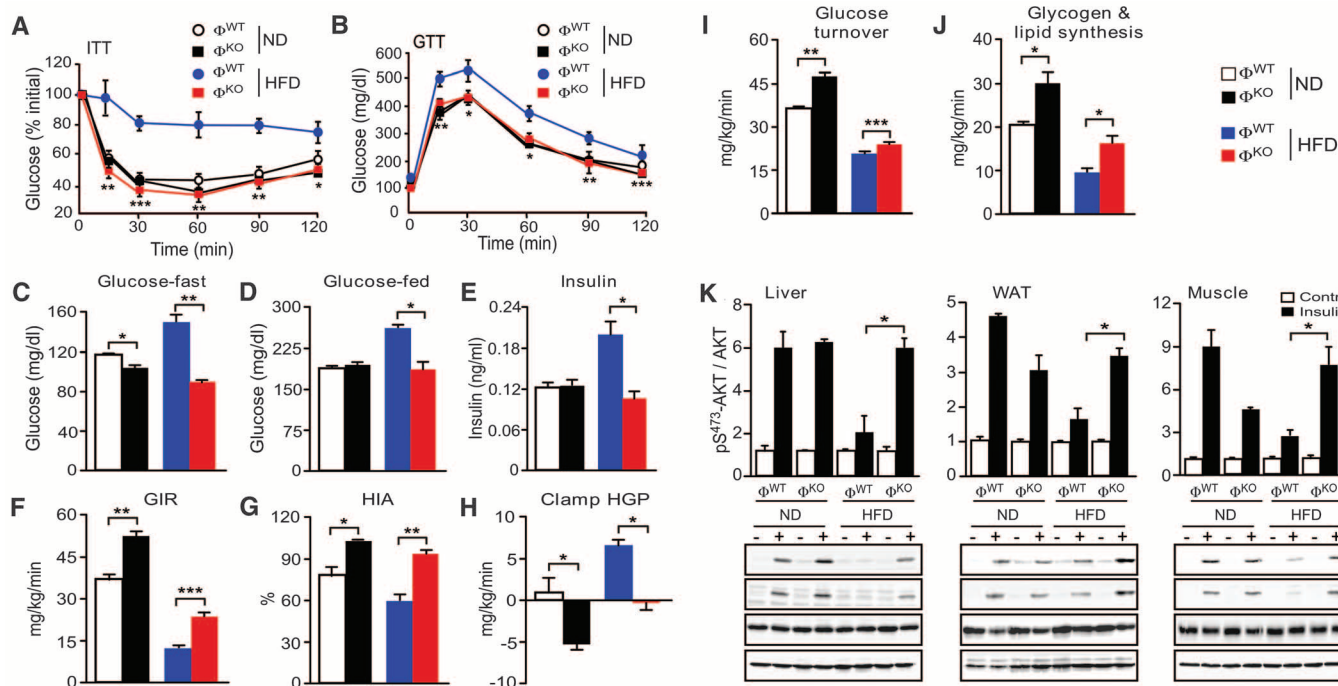


Fig. 1. Macrophage JNK promotes the establishment of obesity-induced insulin resistance. (A) Insulin tolerance tests (ITT) were performed on chow diet and HFD-fed Φ^{WT} and Φ^{KO} mice (4 weeks) by means of intraperitoneal injection of insulin (0.75 U/kg) and measurement of blood glucose concentration (mean $\pm$ SEM; $n = 7 \sim 10$ mice). (B) Glucose tolerance tests (GTT) were performed by means of intraperitoneal injection of glucose (1 g/kg) and measurement of blood glucose concentration (mean $\pm$ SEM; $n = 7 \sim 10$ mice). (C to E) The blood concentration of glucose and insulin in overnight-fasted mice and the blood glucose concentration in fed mice were measured (mean $\pm$ SEM; $n = 10$ mice). (F to J) Insulin sensitivity was measured by using a hyperinsulinemic-euglycemic clamp in conscious mice. The steady-state glucose infusion rate (GIR), hepatic insulin action (HIA), clamp hepatic glucose production (HGP), whole-body glucose turnover, and whole-body

glycogen plus lipid synthesis are presented (mean $\pm$ SEM; $n = 8 \sim 10$ mice). (K) Chow-fed (ND) and HFD-fed mice (4 weeks) were fasted overnight and then treated by means of intraperitoneal injection with 1 U/kg of insulin (15 min). Multiplexed enzyme-linked immunosorbent assay was used to detect Akt and activated (pSer⁴⁷³) Akt in the liver, epididymal adipose tissue (WAT), and gastrocnemius muscle (mean $\pm$ SEM; $n = 3 \sim 5$ mice). Representative tissue samples were also examined by means of immunoblot analysis by probing with antibodies to phospho-Akt, Akt, and α Tubulin (Tub.). Data [(A) to (E)] are representative of three experiments. Data were pooled from [(F) to (J)] eight to ten and [(K) three to five experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ as determined with [(A) and (B)] Student's t test or [(C) to (K)] analysis of variance (ANOVA), with Bonferroni's posttest correction for multiple comparisons.

We performed hyperinsulinemic-euglycemic clamp studies in order to directly assess insulin sensitivity of Φ^{WT} and Φ^{KO} mice in vivo (Fig. 1, F to J). This analysis demonstrated that Φ^{KO} mice exhibited increased whole-body insulin sensitivity because of significant improvements in glu-

cose infusion rates during the clamps (Fig. 1F), hepatic insulin action (Fig. 1G), hepatic glucose production (Fig. 1H), insulin-stimulated whole body glucose turnover (Fig. 1I), and whole-body glycogen plus lipid synthesis (Fig. 1J) as compared with that of Φ^{WT} mice. We also performed

biochemical studies to investigate insulin-stimulated Akt activation in Φ^{WT} and Φ^{KO} mice (Fig. 1K). Feeding a HFD suppressed insulin-stimulated Akt activation in liver, adipose tissue, and muscle of Φ^{WT} mice but not that of Φ^{KO} mice (Fig. 1K). Together, these data demonstrate that HFD-fed

Fig. 2. Macrophage JNK promotes pancreatic islet dysfunction. (A and B) The morphology of pancreatic islets was examined by using chow-fed (ND) and HFD-fed mice (4 weeks) fasted overnight. Sections were stained with antibodies to insulin, glucagon, or F4/80. DNA was stained with 4',6-diamidino-2-phenylindole (blue). Scale bar, 75 μ m. No significant differences between Φ^{WT} and Φ^{KO} islet infiltration by F4/80⁺ macrophages were detected (fig. S19). (C) The islet area per section is presented (mean $\pm$ SEM; $n = 7 \sim 10$ mice). (D) The number of β cells per islet that stained with an antibody to the proliferation marker proliferating cell nuclear antigen (PCNA) is presented (mean $\pm$ SEM; $n = 5 \sim 7$ mice). (E) Glucose-induced insulin release measurements were performed by means of intraperitoneal injection of glucose (2 g/kg) and measurement of blood insulin concentration (mean $\pm$ SEM; $n = 8 \sim 10$ mice). (F) Glucose-induced insulin secretion in vitro. Isolated islets were incubated (1 hour) with low glucose (3.3 mM) or high glucose (16.7 mM). Insulin secretion was measured (mean $\pm$ SEM; $n = 5$ mice). Data [(A) and (B)] are representative of [(A) and (B)] five to ten, (E) three, and (F) two experiments. Data [(C) and (D)] were pooled from five to ten experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ as determined with (E) Student's t test or [(C), (D), and (F)] ANOVA, with Bonferroni's posttest correction for multiple comparisons.

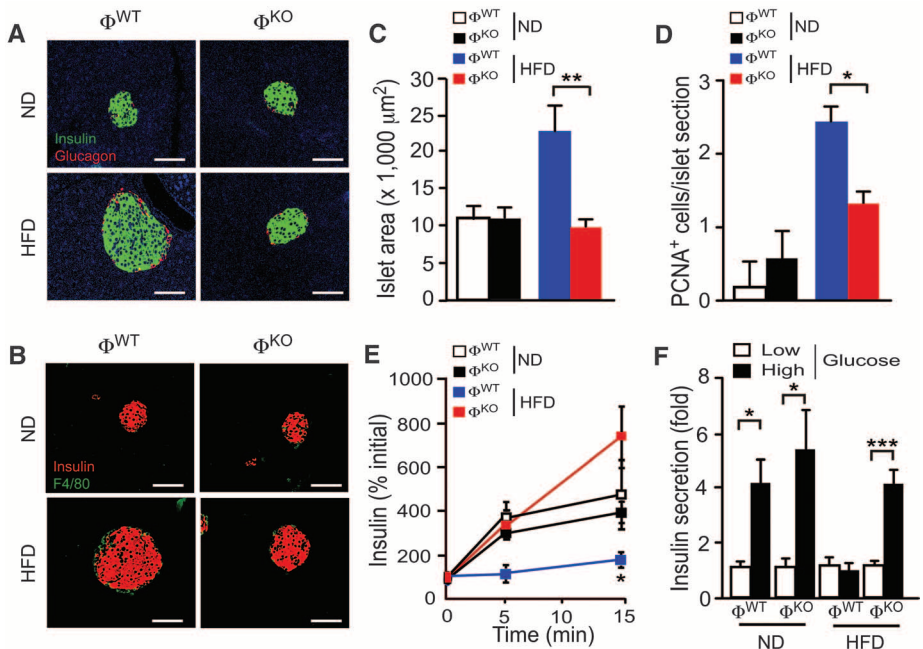
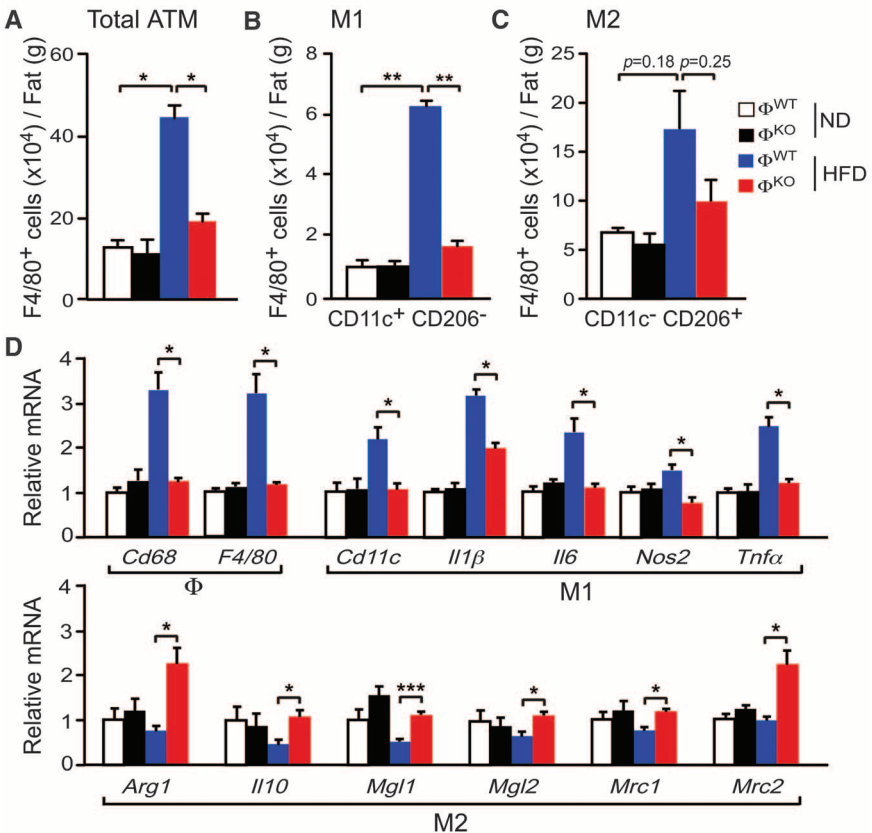


Fig. 3. JNK promotes M1 polarization of adipose tissue macrophages. (A to C) The stromal vascular fraction (SVF) of epididymal adipose tissue was isolated from chow-fed and HFD-fed (4 weeks) mice and examined by means of flow cytometry to detect the total number of F4/80⁺ ATMs, the number of F4/80⁺CD11c⁺CD206[−] (M1 ATMs), and the number of F4/80⁺CD11c[−]CD206⁺ (M2 ATMs) (mean $\pm$ SEM; $n = 5$ mice). (D) Total RNA was isolated from epididymal adipose tissue from chow-fed and HFD-fed Φ^{WT} and Φ^{KO} mice. The relative expression of mRNA associated with M1-polarized macrophages and M2-polarized macrophages was measured by means of quantitative reverse transcription polymerase chain reaction (RT-PCR) assays (mean $\pm$ SEM; $n = 8 \sim 10$ mice). Data [(A) to (C)] are representative of three experiments. Data (D) were pooled from eight to ten experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ as determined with ANOVA, with Bonferroni's post-test correction for multiple comparisons.



Φ^{KO} mice exhibit improved insulin sensitivity as compared with that of HFD-fed Φ^{WT} mice.

Pancreatic islets in chow-fed Φ^{WT} and Φ^{KO} mice were morphologically similar. Feeding a HFD to Φ^{WT} mice, but not Φ^{KO} mice, caused β cell proliferation, islet hypertrophy, and suppression of glucose-stimulated insulin secretion in vivo and in vitro (Fig. 2, A to F). These data are consistent with the observation that HFD-fed Φ^{KO} mice exhibit improved hyperglycemia, hyperinsulinemia, and insulin sensitivity as compared with that of HFD-fed Φ^{WT} mice.

We examined adipose tissue macrophages (ATMs) in Φ^{WT} and Φ^{KO} mice fed a chow or a HFD. No differences in the number of ATMs were detected in Φ^{WT} and Φ^{KO} mice fed a chow diet, whereas feeding a HFD caused an increase in the number of ATMs in Φ^{WT} mice but not Φ^{KO} mice (Fig. 3A and figs. S4 and S6). These data indicate that JNK in macrophages promotes HFD-induced accumulation of ATMs. In contrast, we observed no significant differences in the number of infiltrating eosinophils or neutrophils (figs. S7 and S8).

M1-polarized tissue macrophages are implicated in the development of insulin resistance (6–10). We therefore performed immunophenotyping to examine the populations of ATMs in Φ^{WT} and Φ^{KO} mice. Macrophage-specific JNK-deficiency prevented the accumulation of ATMs

expressing surface markers ($F4/80^{+}CD11c^{+}CD206^{-}$) associated with M1 polarization in adipose tissue of HFD-fed mice (Fig. 3B and fig. S6). In contrast, no significant difference in the accumulation of ATMs expressing surface markers ($F4/80^{+}CD11c^{-}CD206^{+}$) associated with M2 polarization between HFD-fed Φ^{WT} and Φ^{KO} mice was detected (Fig. 3C and fig. S6). These data indicate that JNK expression by macrophages promotes the accumulation of M1-polarized ATMs in HFD-fed mice.

To confirm whether JNK influences the accumulation and polarization of ATMs, we examined adipose-tissue gene expression (Fig. 3D). This analysis demonstrated that macrophage-specific JNK-deficiency decreased the expression of ATM marker genes (*Cd68* and *F4/80*) in adipose tissue of HFD-fed Φ^{KO} mice as compared with that of HFD-fed Φ^{WT} mice. Moreover, gene expression associated with M1 polarization (*Cd11c*, *Il1 β* , *Il6*, *Nos2*, and *Tnf α*) and M2 polarization [*Arg1*, *Il10*, *Mgl1* (*Cd301*), *Mgl2*, *Mrc1* (*Cd206*), and *Mrc2*] demonstrated that macrophage-specific JNK deficiency decreased the expression of genes associated with M1 polarization and increased those associated with M2 polarization in ATMs of HFD-fed mice (Fig. 3D). These data confirm that JNK expression in macrophages promotes both ATM accumulation and M1 polarization in HFD-fed mice.

To further test the role of JNK in the polarization of tissue macrophages, we examined the livers of Φ^{WT} and Φ^{KO} mice (fig. S9). Macrophage-specific JNK deficiency resulted in a decrease in the hepatic expression of tissue macrophage marker genes and genes associated with M1 polarization, whereas expression of genes associated with M2 polarization were increased in HFD-fed Φ^{KO} mice as compared with HFD-fed Φ^{WT} mice (fig. S9C). These data demonstrate that JNK expression by macrophages is required for tissue macrophage accumulation in the liver and polarization to the activated M1 state in HFD-fed mice. The decreased accumulation of M1 tissue macrophages in the liver of HFD-fed Φ^{KO} mice was associated with a marked reduction in hepatic inflammation and gluconeogenesis as compared with that of HFD-fed Φ^{WT} mice (fig. S9, C and D).

The chemokine signaling network is implicated in tissue macrophage function (18). Mice deficient in the chemokine receptors CCR2 or CCR5 have defects in HFD-induced ATM accumulation (7, 19). Immunophenotyping suggests preferential loss of M1 ATMs in *Ccr5*^{−/−} mice (19) and M2 ATMs in *Ccr2*^{−/−} mice (7). Expression of CCR2 ligands (CCL2, CCL7, and CCL8) and CCR5 ligands (CCL3, CCL4, CCL5, and CCL8) was increased when Φ^{WT} mice were fed a HFD (figs. S10A, S11, and S12). In contrast, HFD-induced chemokine expression was

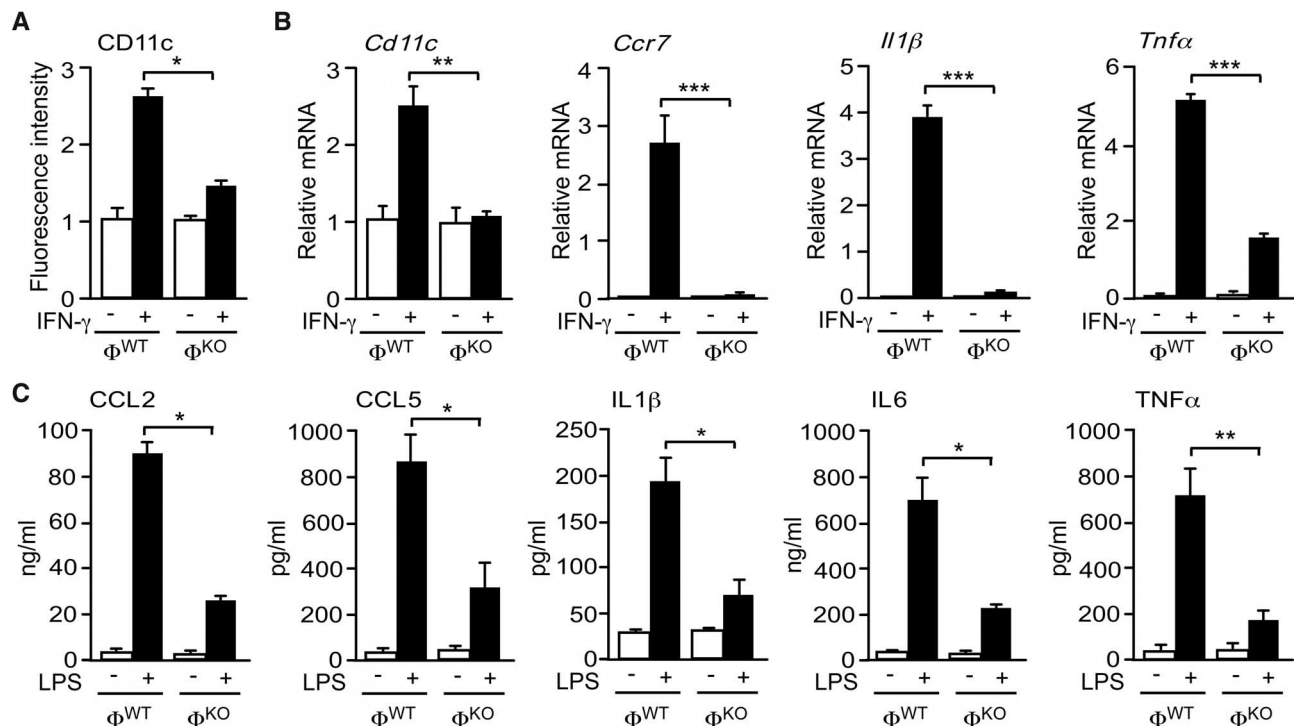


Fig. 4. JNK promotes M1 polarization of macrophages in vitro. (A) BMDMs from Φ^{WT} and Φ^{KO} mice were incubated without or with 100 ng/ml of IFN- γ (36 hours). $F4/80^{+}$ cells were stained with an antibody to the M1 marker CD11c and examined by means of flow cytometry (mean relative fluorescence intensity $\pm$ SEM; $n = 3$ independent experiments). (B) Total RNA was isolated from BMDMs incubated (8 hours) with 100 ng/ml of IFN- γ . The relative expression of the indicated M1 marker genes was measured by means of quantitative

RT-PCR assays (mean $\pm$ SEM; $n = 5$ mice). (C) Chow-fed Φ^{WT} and Φ^{KO} mice were fasted overnight. Blood was collected from the mice 2.5 hours after intraperitoneal injection of LPS (20 mg/kg) or solvent (Control). The blood concentration of CCL2, CCL5, IL1 β , IL6, and TNF α was measured (mean $\pm$ SEM; $n = 10$ mice). Data are representative of (A) three and (C) two experiments. Data (B) were pooled from five experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ as determined with ANOVA, with Bonferroni's posttest correction for multiple comparisons.

largely suppressed in HFD-fed Φ^{KO} mice (figs. S10A, S11, and S12). Moreover, JNK deficiency reduced chemokine expression by macrophages in vitro under a variety of stimulation conditions (figs. S10B and S13 to S15). Because tissue chemokine expression is mediated by both parenchymal cells and macrophages, it is likely that the reduced chemokine expression detected in HFD-fed Φ^{KO} mice is a consequence of both a primary macrophage defect and a secondary response to improved glycemia. Collectively, these data indicate that reduced chemokine signaling may contribute to the decreased accumulation of ATMs in Φ^{KO} mice. However, no selective defect in the expression of CCR2 or CCR5 receptors or ligands was detected in Φ^{KO} mice compared with Φ^{WT} mice. The loss of M1 ATMs in Φ^{KO} mice may therefore represent an intrinsic defect in M1 polarization rather than inefficient M1 ATM recruitment.

To test the requirement of JNK for M1 macrophage function in vitro, we isolated bone marrow-derived macrophages (BMDMs) from Φ^{WT} and Φ^{KO} mice (Fig. 4). Treatment with IFN- γ causes M1 differentiation (3) and expression of the cell-surface marker CD11c (20). Macrophage-specific JNK deficiency caused reduced M1 differentiation as compared with that in control mice (Fig. 4A). Furthermore, JNK deficiency caused decreased expression of M1 marker genes (*Ccr7* and *Cd11c*), chemokines (*Ccl2* and *Ccl5*), and cytokine genes (*Il1 β* , *Il6*, and *Tnf α*) in IFN- γ -stimulated macrophages (Fig. 4B and fig. S14). Similar defects were detected in lipopolysaccharide (LPS)-stimulated macrophages (fig. S15). Moreover, the blood concentration of M1-associated cytokines and chemokines was significantly reduced in LPS-treated Φ^{KO} mice as compared with Φ^{WT} mice in vivo

(Fig. 4C). Together, these data demonstrate that JNK deficiency suppresses M1 polarization. In contrast, no significant difference in M2 differentiation between BMDMs isolated from Φ^{WT} and Φ^{KO} mice was detected (figs. S16 to S18).

The observation that JNK is required for the differentiation of pro-inflammatory macrophages provides an explanation, in part, for previous findings that have implicated JNK in inflammatory responses (17), including the promotion of T_H1 immune responses (21–23). JNK-dependent inflammatory macrophages in the liver may account for the requirement of JNK for the development of fulminant hepatitis in mouse models (24). JNK-dependent tissue macrophages may also contribute to the inflammation associated with insulin resistance caused by obesity. Indeed, JNK in macrophages is required for the accumulation of inflammatory tissue macrophages and insulin resistance caused by diet-induced obesity. Drug-mediated targeting of macrophage-expressed JNK therefore represents a potential therapeutic approach to suppress inflammation that may be applicable to the treatment of inflammatory disorders.

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Supplementary Materials

www.sciencemag.org/cgi/content/full/science.1227568/DC1
Materials and Methods
Figs. S1 to S19
References (25–30)

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Influence of Threonine Metabolism on S-Adenosylmethionine and Histone Methylation

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Threonine is the only amino acid critically required for the pluripotency of mouse embryonic stem cells (mESCs), but the detailed mechanism remains unclear. We found that threonine and S-adenosylmethionine (SAM) metabolism are coupled in pluripotent stem cells, resulting in regulation of histone methylation. Isotope labeling of mESCs revealed that threonine provides a substantial fraction of both the cellular glycine and the acetyl-coenzyme A (CoA) needed for SAM synthesis. Depletion of threonine from the culture medium or threonine dehydrogenase (Tdh) from mESCs decreased accumulation of SAM and decreased trimethylation of histone H3 lysine 4 (H3K4me3), leading to slowed growth and increased differentiation. Thus, abundance of SAM appears to influence H3K4me3, providing a possible mechanism by which modulation of a metabolic pathway might influence stem cell fate.

Connections between pluripotency and the metabolic state of mouse embryonic stem cells (mESCs) are only beginning to be

described (1). Metabolically, mESCs are characterized by a distinct mode of amino acid catabolism driven by the enzyme that catalyzes threonine

(Thr) oxidation, threonine dehydrogenase (Tdh) (1). The abundance of Tdh in mESCs is more than 200 times that in mouse embryonic fibroblasts (MEFs), and Thr restriction or Tdh inhibition abolishes mESC growth (1–3). Thus, Thr oxidation appears to be critical for mESCs, but the relationship between downstream metabolites and the pluripotent state remains unclear.

To investigate how metabolism is altered upon reprogramming to the pluripotent state,

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we used liquid chromatography–based tandem mass spectrometry (LC-MS/MS) (4) to profile metabolomic changes during reprogramming into induced pluripotent stem cells (iPSCs). We used MEFs transfected with doxycycline-activated transgenes encoding Oct4, Sox2, Klf4, and Myc (iOSKM) (2, 5) and profiled metabolism during reprogramming. Hierarchical clustering based on the abundance of each metabolite revealed that iPSCs were similar to mESCs and were distinct from MEFs (Fig. 1, A and B). Inspection of the top metabolites regulated by OSKM-induced reprogramming revealed a large number of glycolytic intermediates (fig. S1A and table S1), many of which were significantly more abundant within just 4 days of reprogramming (Fig. 1C), when proliferation was accelerated but the cells were not yet pluripotent (2). Thus, increases in glycolytic intermediates occurred before the acquisition of pluripotency (Fig. 1C). The other top metabolites regulated during reprogramming are involved in Thr and S-adenosylmethionine (SAM) metabolism. In contrast to glycolytic intermediates, Thr- and SAM-related metabolites changed late in reprogramming, which suggests that enhanced Thr and SAM metabolism ac-

companied acquisition of the pluripotent state (Fig. 1D). Upon reprogramming, some of the largest decreases were in Thr, Cys, and folate, and the largest increases were in SAM and cystathionine.

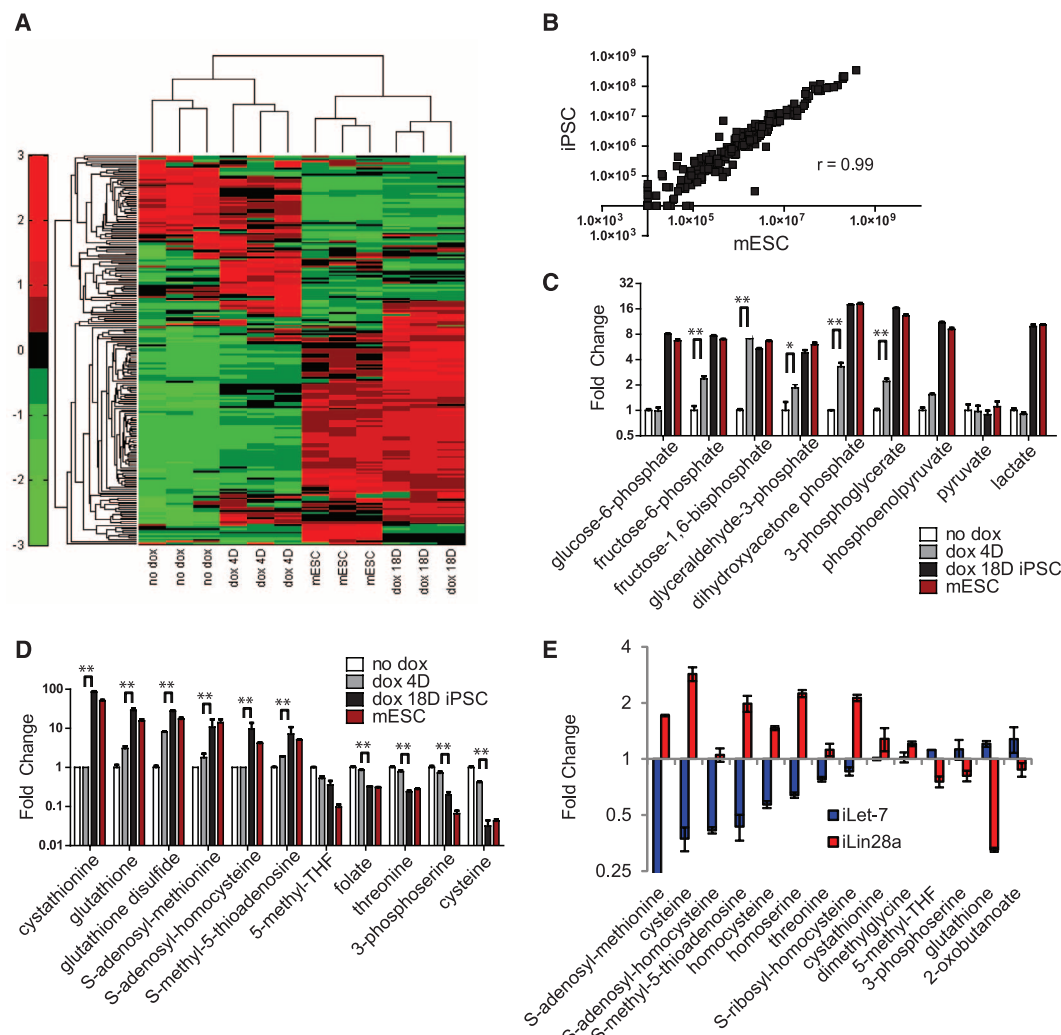
Metabolism is also rewired during mESC differentiation. In mESCs, the *let-7* microRNA promotes differentiation by suppressing the pluripotency network, whereas *Lin28a* promotes pluripotency by repressing *let-7* (6, 7). Hence, we profiled metabolic changes in mESCs acutely after dox-induced activation of a transgene encoding *let-7* or *Lin28a* (iLet-7 or iLin28a) while the cell cycle remained unperturbed (fig. S1B). *Lin28a* and *let-7* regulated 38 metabolites in a reciprocal manner (fig. S1C), among which were a large number of Thr and SAM metabolites (Fig. 1E); these findings provide further support for the idea that Thr and SAM metabolism are coupled to pluripotency.

We integrated our metabolomics data on mESCs (table S1) with cDNA microarray data on mESCs (2) using the KEGG database of metabolic networks. This analysis showed that many of the metabolic enzymes that channel Thr metabolism into SAM metabolism (e.g., Tdh,

Gcat, Glc6p, Dhfr, Foli1r, Mat2a/b, and Ahcy) are more abundant in mESCs than in MEFs (Fig. 2A). Consistent with the enzyme expression patterns, several Thr-SAM pathway inputs such as Thr, Cys, and folate were less abundant in mESCs than in MEFs, whereas downstream outputs such as SAM and cystathionine were more abundant in mESCs than in MEFs (Fig. 1D). Thus, a Thr-SAM pathway appears to be activated in mESCs.

To test this pathway, we traced the metabolic fate of [^{14}C]Thr in mESCs with high-performance liquid chromatography (HPLC). ^{14}C was incorporated into Gly and Glu, indicating that Thr was used to synthesize these amino acids (Fig. 2B). In contrast, MEFs incubated with [^{14}C]Thr did not exhibit Thr catabolism (fig. S2A). We also traced the fate of [^{13}C]Thr in mESCs with LC-MS/MS metabolomics (fig. S2, B to F, and table S1). mESCs used Thr to synthesize acetyl-CoA–derived tricarboxylic acid (TCA) cycle intermediates (Fig. 2, C and D). At steady state, [^{13}C]Thr contributed ~20% of the citrate via acetyl-CoA, whereas [^{13}C]glucose contributed ~35% via acetyl-CoA (+2 isotopomer). Thus, Thr contributes significantly to the acetyl-CoA

Fig. 1. The Thr-SAM pathway is activated by pluripotency factors. (A) Heat map showing relative abundance of metabolites in iOSKM-MEFs in the absence of doxycycline treatment (no dox); 4 days after dox (dox 4D); 18 days after dox, whereupon iPSC clones are fully reprogrammed (dox 18D); and mESCs, normalized to total biomass ($n = 3$), and measured using selected reaction monitoring (SRM) and LC-MS/MS. (B) Scatterplot of metabolite intensities in mESCs (x axis) versus iPSCs (y axis). Metabolite intensities were obtained from the integrated total ion current from a single SRM transition. (C) SRM analysis of abundance in glycolytic intermediates in iOSKM-MEFs during reprogramming ($n = 3$). $^*P < 0.05$, $^{**}P < 0.01$. (D) SRM analysis of abundance in Thr-SAM pathway intermediates in iOSKM-MEFs during reprogramming ($n = 3$). $^{**}P < 0.01$. (E) SRM analysis of abundance in Thr-SAM pathway intermediates in iLet-7 and iLin28a mESCs after dox ($n = 3$). All error bars represent SEM from three independent measurements.



pool in mESCs (Fig. 2D). [U-¹³C]Thr-derived Gly also donated its ¹³C-methyl group to ultimately generate 5-methyltetrahydrofolate (5mTHF) and SAM (+1 isotopomer), whereas [U-¹³C]Ser-derived Gly contributed little to the synthesis of these metabolites (Fig. 2, C and D). Although only ~25% of intracellular Gly and 5mTHF, and ~10% of SAM, were labeled by [U-¹³C]Thr at steady state, these numbers underestimate the contribution of Thr to the synthesis of these intermediates, because of rapid one-for-one antiport of intracellular [¹³C]Gly and [¹³C]Met for extracellular [¹²C]Gly and [¹²C]Met. Evidence for rapid antiport-mediated ¹³C dilution was provided by the appearance of large quantities of [¹³C]Gly and

[¹³C]Met in the media: Within 1 hour of [¹³C]Thr addition to the media, 6% of extracellular Gly and 13% of extracellular Met was ¹³C-labeled, with no substantial consumption of Gly or Met from the media (fig. S2G). These data suggest that the rapid conversion of Thr to Gly by Tdh provides a major fraction of the 5mTHF needed for recycling S-adenosylhomocysteine (SAH) to SAM. To test whether Thr was indeed a major fuel source for Gly and SAM metabolism, we profiled metabolic changes upon Thr restriction in mESCs; all the Thr in Dulbecco's modified Eagle's medium (DMEM) was removed, but there remained enough Thr in the serum for cellular protein synthesis (1, 8). Thr restriction affected mESC

viability additively with, and thus independently of, protein synthesis (fig. S2, H and I). The 80% drop in intracellular Thr caused intracellular Gly and the SAM/SAH ratio to decrease steadily, suggesting an imbalance in Gly synthesis and consumption (Fig. 2, E and F, and table S1). In contrast, glucose-6-phosphate, fructose-6-phosphate, lactate, and the ATP/AMP (adenosine triphosphate/adenosine monophosphate) ratio increased (Fig. 2, E and F), consistent with a compensatory increase in glycolysis. However, some of the TCA cycle intermediates still decreased (fig. S2J). The drop in NADH (reduced nicotinamide adenine dinucleotide) might indicate a drop in Thr-derived acetyl-CoA, which fuels the TCA cycle's production

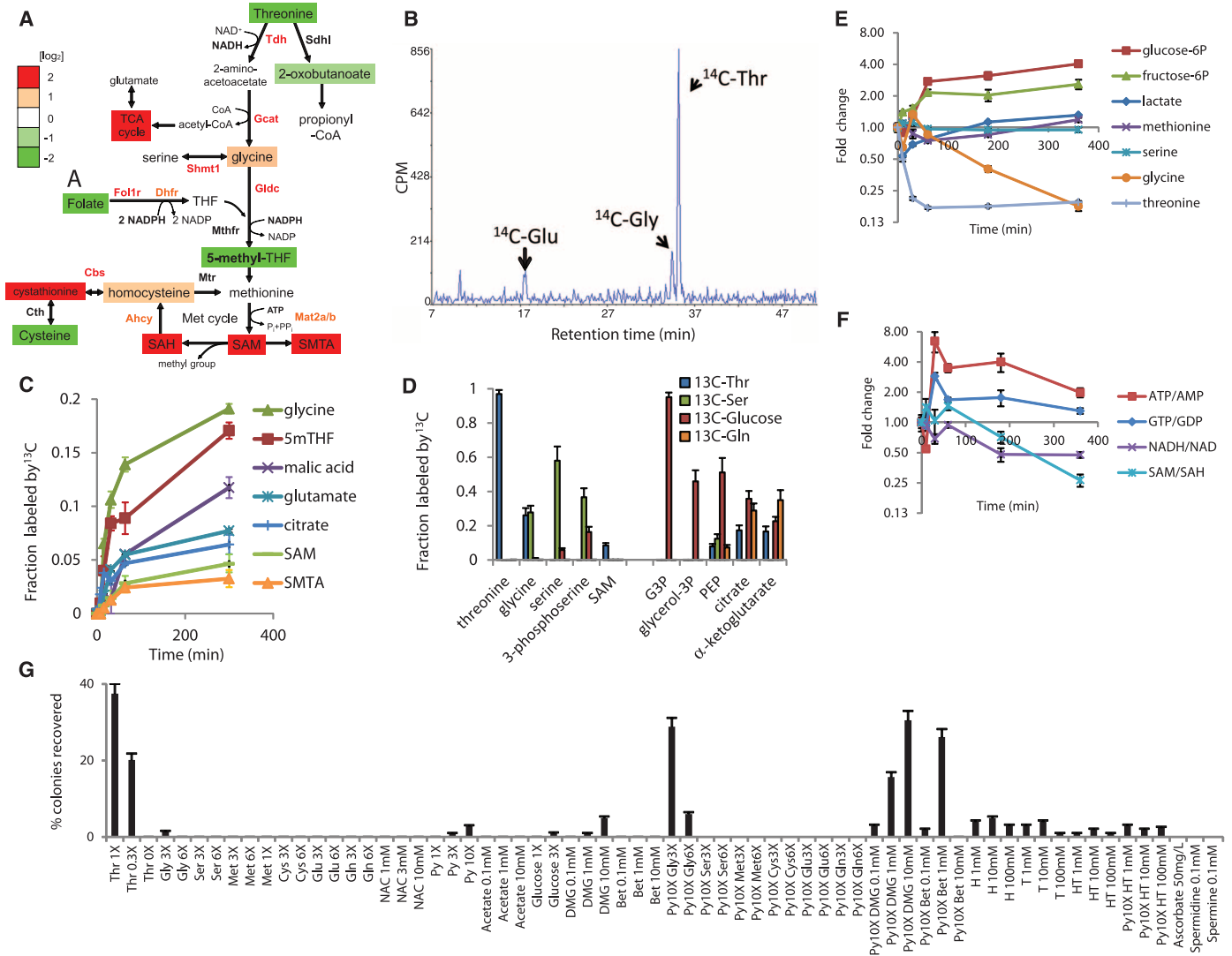


Fig. 2. Thr is catabolized to maintain the SAM/SAH ratio in mESCs. (A) Schematic of Thr-SAM pathway activity in pluripotent stem cells, relative to MEFs. (B) HPLC analysis of ¹⁴C-labeled amino acids derived from [U-¹⁴C]Thr in mESCs after 24 hours. Scintillation counts per minute (CPM) are plotted against retention time. (C) Fraction of intracellular metabolites derived from [U-¹³C]Thr in mESCs over 5 hours, as measured by SRM analysis (*n* = 3). (D) Steady-state fraction of intracellular metabolites derived from [U-¹³C]Thr, [U-¹³C]Ser, [U-¹³C]glucose, or [U-¹³C]Gln in mESCs after 48 hours, as measured by SRM analysis (*n* = 3). (E) SRM analysis of metabolite abundances in mESCs during

6 hours of Thr restriction, relative to time zero (*n* = 3). (F) SRM analysis of several metabolic ratios over a 6-hour time course, relative to time zero (*n* = 3). (G) Feeder-free mESCs were subjected to Thr restriction for 12 hours, then supplemented for 36 hours with the indicated metabolites at the given concentrations. Alkaline phosphatase-positive colonies were quantified and normalized to mESC colony numbers in normal mESC media (% colonies recovered). X denotes relative concentration with respect to DMEM. NAC, N-acetylcysteine; Py, pyruvate; DMG, dimethylglycine; Bet, betaine; H, hypoxanthine; T, thymidine. All error bars represent SEM from three independent measurements.

of reducing equivalents (Fig. 2F and fig. S2, K and L). Thus, our results support the idea that Thr is critically required in mESCs because of its contribution to Gly for 5mTHF and SAM synthesis, and because acetyl-CoA produced from Thr could contribute to this anabolic process (Fig. 2A).

As another test of this model, we attempted to suppress the effects of Thr restriction on mESCs by supplementing the culture medium with downstream metabolites in the Thr-SAM pathway. Addition of Gly and pyruvate (a source of acetyl-CoA), but neither alone, prevented mESC death after Thr restriction (Fig. 2G). Glucose, acetate, Ser, Cys, Glu, Gln, *N*-acetylcysteine, ascorbate, or combinations with pyruvate all failed to prevent mESC death after Thr restriction. However, a combination of pyruvate with the methyl donors dimethylglycine or betaine prevented death from Thr restriction (Fig. 2G); these results suggest that Thr-derived 5mTHF and its main product SAM (Fig. 2, C and D) are essential for mESCs. SAM could not be used because it

cannot cross mammalian plasma membranes (9). In contrast, 3-deazaadenosine (DZA) (10), which decreases the SAM/SAH ratio by inhibiting SAH hydrolase, did enter mESCs and inhibited mESC viability (fig. S2M). Hypoxanthine and thymidine also failed to prevent mESC death after Thr restriction although they could enter mESCs (11), which suggests that nucleosides are not important downstream products of Thr-derived Gly. A downstream product of SAM catabolism, the polyamine spermidine (12), was also ineffective. Thus, Thr appears to be necessary to generate the optimal balance of Gly and acetyl-CoA required to synthesize 5mTHF for maintaining the SAM/SAH ratio in mESCs.

SAM is the universal substrate for all protein methylation reactions in the cell, and the SAM/SAH ratio is important for regulating protein methylation because methyltransferases are product-inhibited by SAH (Fig. 3A) (13). To test whether Thr restriction affects protein methylation, we used a pan-methyl-lysine antibody to

detect differential lysine methylation of proteins after restriction of Thr, Gly, and Ser. Among the most abundant methylated proteins (14), histone H3 showed a drop in methylation upon Thr restriction, whereas heat shock protein 8, elongation factor-1 α , and actin showed only subtle changes (Fig. 3B). Because euchromatin is crucial for epigenetic plasticity in mESCs, and because histone H3 lysine 4 trimethylation (H3K4me3) and H3 acetylation (H3ac) are important for maintaining euchromatin (15–18), we tested whether Thr catabolism regulates H3K4me3 and H3ac in mESCs. Under milder conditions of Thr restriction (0.3X) that did not inhibit mESC growth (fig. S3A), H3K4me3 dropped after 48 hours, whereas H3ac remained unchanged (Fig. 3C). In comparison, MEFs showed no changes in H3K4me3 nor H3ac upon 0.3X Thr restriction (Fig. 3C), consistent with our observations that Thr-SAM metabolism is activated only in the pluripotent state.

To test the sensitivity and reversibility of H3K4me3, we supplemented Thr-restricted mESCs with various doses of Thr. Although mESCs lost most of their H3K4me3 after just 6 hours of Thr restriction, refeeding with various concentrations of Thr for 6 hours reversed the drop in H3K4me3 (fig. S3B). H3K4me3 could also be restored by adding pyruvate and Gly to the medium (Fig. 3D). Both pyruvate and Gly were necessary to restore the SAM/SAH ratio to a normal state, consistent with the idea that Thr-derived acetyl-CoA and Gly regulate H3K4me3 by modulating the SAM/SAH ratio (Fig. 3E). Conversely, DZA, which decreases the SAM/SAH ratio, led to a rapid extinction of H3K4me3 and overrode the effects of pyruvate and Gly on mESCs (Fig. 3, D and E, and fig. S3C), which suggests that acetyl-CoA and Gly regulate the SAM/SAH ratio to modulate H3K4me3. We then tested H3 methylation on lysines 4, 9, 27, 36, and 79 in mESCs after restriction of a variety of individual amino acids. H3K4me3 and H3K4me2 were decreased by Thr restriction. H3K4me1, H3K9me3, H3K27me3, H3K36me3, and H3K79me3 did not change significantly with any amino acid (Fig. 3F). Also, α -ketoglutarate did not change significantly during Thr restriction (fig. S2J), suggesting minimal influence on α -ketoglutarate-dependent histone demethylases. These results suggest that the SAM/SAH ratio maintained by Thr catabolism is not required to regulate all protein lysine methylation, but rather certain specific lysines such as H3K4me2 and H3K4me3 in mESCs (Fig. 3F). Assays of total H3K4 methyltransferase activity from nuclear lysates further indicated that the change in H3K4me3 with Thr concentration was not due to a change in the net amount of methyltransferase activity (fig. S3D), supporting the idea that it is the change in the substrate/product ratio (SAM/SAH) that influenced the amount of H3K4me3.

To test whether the Thr-SAM pathway has functional consequences on the pluripotency and differentiation of mESCs, we partially depleted

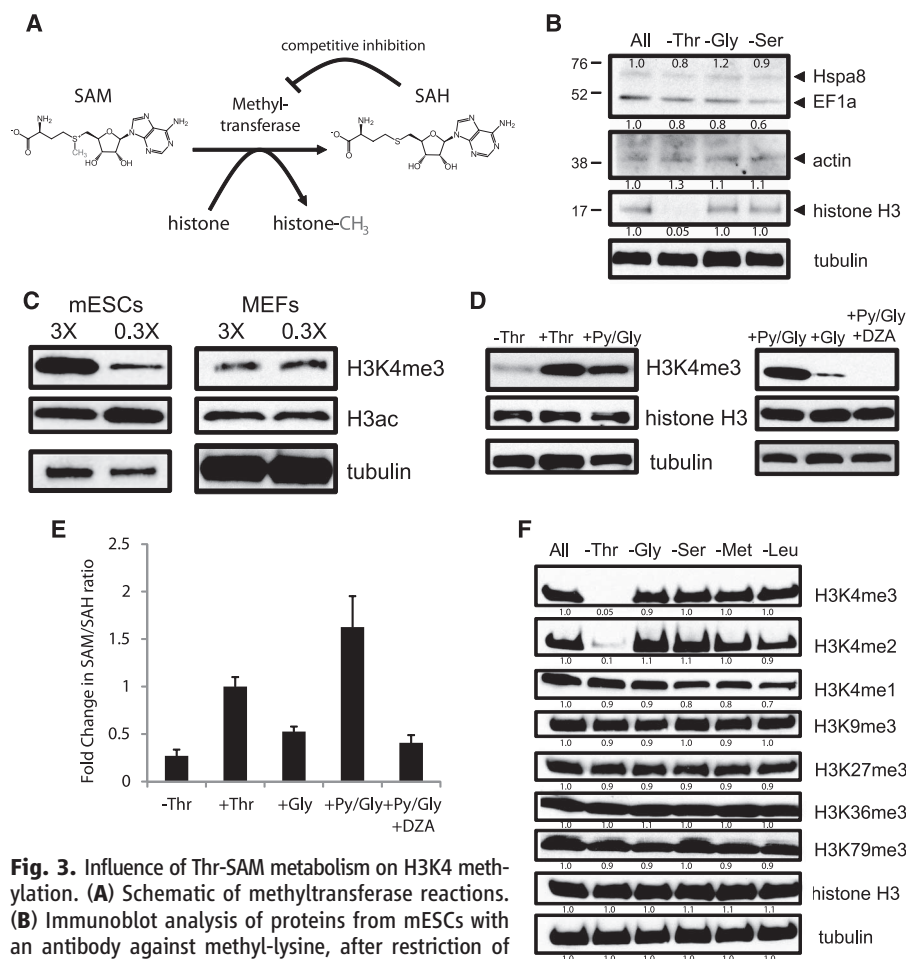


Fig. 3. Influence of Thr-SAM metabolism on H3K4 methylation. (A) Schematic of methyltransferase reactions. (B) Immunoblot analysis of proteins from mESCs with an antibody against methyl-lysine, after restriction of the indicated amino acids for 24 hours. (C) Immunoblot analysis of mESCs and MEFs for H3K4me3 and H3ac when exposed to 3X and 0.3X concentrations for 48 hours. (D) Immunoblot analysis of mESCs for H3K4me3 after Thr restriction (0X) for 6 hours, then re-fed for 6 hours with indicated metabolites. (E) SAM/SAH ratio after Thr restriction (0X) for 6 hours, then re-fed for 6 hours with indicated metabolites. (F) Immunoblot analysis of mESCs for H3K4me3, H3K4me2, H3K4me1, H3K9me3, H3K27me3, H3K36me3, and H3K79me3 after restriction (0X) of the indicated amino acids for 24 hours.

the Tdh enzyme by RNA interference (Fig. 4A and fig. S4A). In low-Thr conditions, partial Tdh depletion led to a decrease in mESC growth and an increase in mESC differentiation (Fig. 4B). Expression profiling by quantitative reverse transcription polymerase chain reaction (qRT-PCR) revealed that Tdh depletion led to a decrease in expression of pluripotency factors, including *Oct4*, *Sox2*, *Nanog*, *Rex1*, and *Blimp1*, and increased expression of the differentiation factors *Foxa2* and *Sox17* (fig. S4B). Embryoid body differentiation of the mESCs for 3 days showed that Tdh depletion led to more rapid extinction of the pluripotency factor *Sox2* and an aberrant increase in transcription of the differentiation factors *Gata4* and *Sox17* (fig. S4C).

The differentiation of mESCs after partial Tdh depletion was dependent on the amount of Thr in the culture medium, which suggests that Tdh was required for its metabolic function (Fig. 4B). Indeed, Tdh depletion decreased Thr-SAM flux (Fig. 4C). At steady state, Tdh depletion also decreased Gly, citrate, and the SAM/SAH ratio (Fig. 4D). Tdh depletion also decreased H3K4me3 abundance (Fig. 4A). Thus, Thr catabolism by Tdh appears to be required for maintenance of the pluripotent epigenetic state. We also tested whether enzymes downstream of Tdh in the Thr-SAM pathway are also required by mESCs, including glycine decarboxylase

(Gldc), which produces methylene-THF from Gly (19), and methionine adenosyltransferase (Mat2a), which produces SAM from Met. Depletion of Gldc decreased Thr-SAM flux and the level of H3K4me3, and decreased mESC colony growth (fig. S4, D to H). In contrast, transient overexpression of Gldc prevented mESC death after Thr restriction (fig. S4, I and J), suggesting that the 5mTHF and SAM downstream of Thr and Gly are necessary for mESCs. Depletion of the downstream Mat2a also decreased SAM synthesis and H3K4me3 levels, and decreased mESC colony growth (fig. S4, F to H). Conversely, transient overexpression of the SAH hydrolase (Ahcy), which decreases SAH and thereby increases the SAM/SAH ratio, prevented mESC death after Thr restriction (fig. S4, I and J). Finally, components of the H3K4 methyltransferase complexes—such as Wdr5, Dpy30, and Setd1a—also improved mESC survival after Thr restriction, supporting the idea that Thr-SAM metabolism may affect mESCs in part through effects on H3K4me3.

Our results show that the Thr-SAM pathway is activated in mouse pluripotent stem cells. Thr-dependent changes in the SAM/SAH ratio correlated with H3K4me3, thus revealing a possible mechanistic link between cellular metabolism and the epigenetic state. The unique activity of Tdh in converting Thr into both Gly and acetyl-

CoA appears to optimize the synthesis of SAM to maintain a high SAM/SAH ratio. H3K4me3 was also more sensitive than methylation of other histone H3 lysines to changes in Thr metabolism, possibly because of the high abundance of this H3 methylation mark in the euchromatin of mESCs and its rapid turnover (15–18, 20, 21). H3K4me3 is critical for self-renewal of pluripotent stem cells (22). Because dysregulation of Gly metabolism has been implicated in a variety of human cancers (4, 19, 23, 24) and because the upstream TDH enzyme has been lost by mutation in humans (1), our findings may provide insight into a metabolic pathway that is dysregulated during human tumorigenesis.

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Supplementary Materials

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Figs. S1 to S4

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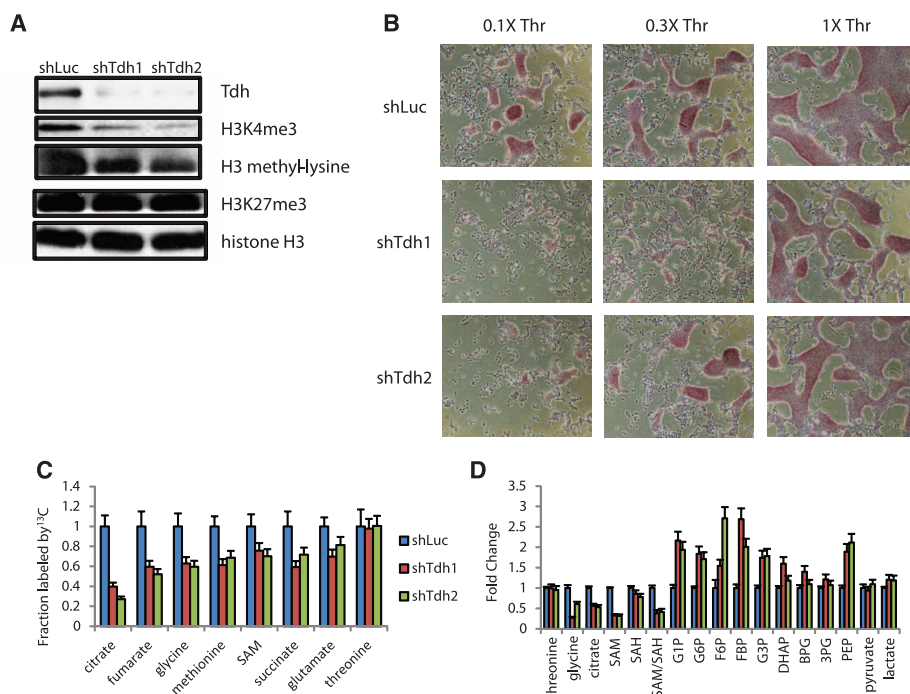


Fig. 4. Tdh regulates the pluripotency of mESCs. **(A)** Immunoblot for Tdh, H3K4me3, H3K27me3, and pan-methyl-lysine in mESCs grown under 0.1X Thr, immediately after depletion of Tdh with two different short hairpin RNAs (shTdh), compared to a control shRNA targeting luciferase (shLuc). **(B)** Micrographs of alkaline phosphatase staining in mESCs after shTdh or shLuc, seeded at clonal density without feeder MEFs, after 48 hours of culture in 0.1X, 0.3X, and 1X Thr concentrations. **(C)** Steady-state fraction of intracellular metabolites derived from [U-¹³C]Thr in mESCs after 24 hours, as measured by SRM analysis, after 48 hours of dox induction of shTdh or shLuc ($n = 3$). **(D)** SRM analysis of metabolite abundances in mESCs after 48 hours of dox induction of shTdh or shLuc ($n = 3$). All error bars represent SEM from three independent measurements.

Natively Inhibited *Trypanosoma brucei* Cathepsin B Structure Determined by Using an X-ray Laser

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The *Trypanosoma brucei* cysteine protease cathepsin B (TbCatB), which is involved in host protein degradation, is a promising target to develop new treatments against sleeping sickness, a fatal disease caused by this protozoan parasite. The structure of the mature, active form of TbCatB has so far not provided sufficient information for the design of a safe and specific drug against *T. brucei*. By combining two recent innovations, in vivo crystallization and serial femtosecond crystallography, we obtained the room-temperature 2.1 angstrom resolution structure of the fully glycosylated precursor complex of TbCatB. The structure reveals the mechanism of native TbCatB inhibition and demonstrates that new biomolecular information can be obtained by the “diffraction-before-destruction” approach of x-ray free-electron lasers from hundreds of thousands of individual microcrystals.

Over 60 million people are affected by human African trypanosomiasis (HAT), also known as sleeping sickness, which causes ~30,000 deaths per year (1). The protozoan parasite *Trypanosoma brucei*, transmitted by tsetse flies, infects the blood and the lymphatic system before invading the brain. Severe clinical manifestations occur within weeks or months. Current treatments of HAT rely on antiparasitic drugs developed during the last century, without knowledge of the biochemical pathways. These treatments are limited in their efficacy and safety, and drug resistance is increasing (2–4). Thus, new compounds that selectively inhibit vital pathways of the parasite without adverse affects to the host are urgently required. A promising strategy is to target lysosomal papainlike cysteine proteases that are involved in host-protein degradation, such as cathepsin B (5). The knockdown of this essential enzyme in *T. brucei* resulted in clearance of parasites from the blood of infected mice and cured the infection (6), which qualify cathepsin B as a suitable drug target. Cysteine proteases are synthesized as inactive precursors with N-terminal propeptides that act as potent and selective intrinsic inhibitors until the proteases enter the lysosome (7), where the propeptide is released and forms the mature active enzyme. Such native propeptide-inhibited structures have been used to develop species-specific protease inhibitors against proteases of other *Trypanosoma* species, e.g., cruzipain of *T. cruzi* (causing human Chagas disease in America) and congopain of *T. congolense* (causing nagana in cattle) (8, 9). This approach could not be explored for *T. brucei*

cathepsin B (TbCatB) because of the lack of structural information on the mode of propeptide inhibition and the large extent of structural conservation at the active site between mammalian and trypanosome cathepsin B (10–12). Previously solved mature *T. brucei* and human CatB structures show differences at the S2 and in part of the S1' subsite of the substrate-binding cleft (Fig. 1C) and have been suggested as possible targets for the development of species-specific CatB inhibitors (10). Together with the natively inhibited human procathepsin B structure (13), our work fills the gap to understand the structural basis for species-specific inhibition.

The growth of large well-ordered protein crystals is one of the major bottlenecks in structure determination by x-ray crystallography—with important biological targets, such as integral membrane proteins and posttranslationally modified proteins, proving particularly challenging to crystallize (14). Sizable crystals are required to obtain measurable high-resolution diffraction data within an exposure that is limited by the accumulation of radiation damage (15). Although microfocus beamlines enable the collection of diffraction data from micron-sized protein crystals (16), the tolerable dose limit of less than 30 MGy for cryogenically cooled protein crystals remains, which limits the achievable signal. The tolerable dose for room temperature measurements is about 1 MGy (15). We have previously shown that micron-sized crystals of glycosylated TbCatB spontaneously form in insect cells during protein overexpression (11). Such crystals are extremely well suited for the new method of serial femtosecond

crystallography (SFX) (17). X-ray free-electron laser (FEL) pulses of less than 100-fs duration allow the dose to individual crystals to exceed the ~1 MGy limit by over a thousand times because of the “diffraction-before-destruction” principle (17, 18). Diffraction data are recorded for each pulse as crystals are continually replenished by a microcrystal suspension in aqueous buffer flowing across the FEL beam in a vacuum in a fine liquid jet.

The Coherent X-ray Imaging (CXI) beamline (19) at the Linac Coherent Light Source (LCLS) enables high-resolution data collection using the SFX approach (20). We used this instrument to obtain diffraction data from in vivo grown crystals of TbCatB produced in the baculovirus-infected *Spodoptera frugiperda* (baculovirus-Sf9) insect cell system (11) (Fig. 1, A and B). Crystals with average dimensions of about 0.9 by 0.9 by 11 μm^3 (fig. S1) were sent in a 4- μm -diameter column of buffer fluid at room temperature, at a flow rate of 10 $\mu\text{l}/\text{minute}$, by using a liquid microjet (21). X-ray pulses from the FEL were focused onto this column to a spot 4 μm in diameter, before the breakup of the jet into drops (fig. S2). Single-pulse diffraction patterns of randomly oriented crystals that, by chance, were present in the interaction region, were recorded at a 120-Hz repetition rate by a Cornell-SLAC pixel array detector (CSPAD) (19, 20) at 9.4-keV photon energy (1.3 Å wavelength). An average pulse energy of 0.6 mJ at the sample (4×10^{11} photons per pulse) with a duration of less than

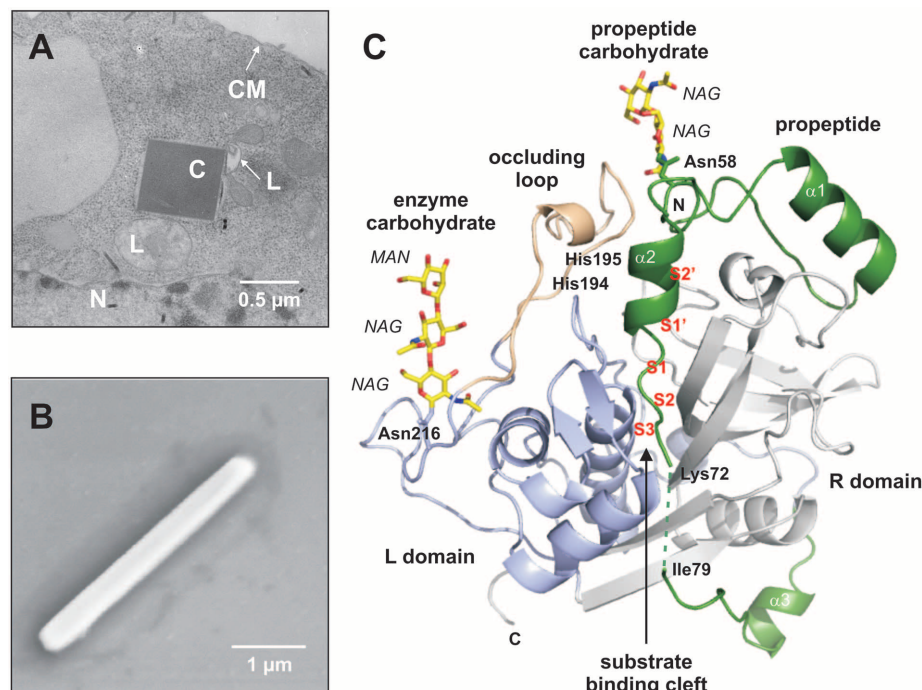
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Fig. 1. In vivo grown crystals and three-dimensional structure of the TbCatB-propeptide complex. (A) Transmission electron microscopy (EM) of an infected Sf9 insect cell showing a crystal of overexpressed TbCatB inside the rough endoplasmic reticulum that is cut perpendicular to its long axis. N, nucleus; L, lysosome; C, crystal; CM, cell membrane. (B) Scanning EM of a single TbCatB crystal after isolation. (C) Cartoon plot of the TbCatB-propeptide complex exhibiting the typical papain-like fold of cathepsin B-like proteases (supplementary text S1). Gray, R domain; blue, L domain; beige, occluding loop. The native propeptide (green) blocks the active site. The subsites of the substrate-binding cleft N-terminal (nonprime: S2, S3) and C-terminal (prime: S1', S2') to the active site (S1) have been identified by comparison with the human CatB structure (13) and labeled (red) according to Schechter and Berger (27). Two N-linked carbohydrate structures (yellow) consist of *N*-acetylglucosamine (NAG) and mannose (MAN) residues (yellow, carbon atoms; blue, nitrogen atoms; red, oxygen atoms).



40 fs gave an x-ray intensity above 10^{17} W/cm² and a maximum dose of about 31 MGy per crystal. This dose exceeds that tolerable at room temperature with conventional data collection approaches because of the radically different time scales and dose rates. The electron and photon beam parameters are summarized in table S1. Almost 4 million individual “snap-shot” diffraction patterns were collected. Of these, 293,195 snapshots contained crystal diffraction (fig. S3), from which 178,875 (61%) diffraction patterns were indexed and combined into a three-dimensional data set of structure factors by “Monte Carlo” integration of partial reflections from each randomly oriented microcrystal (22, 23). The resulting complete set of structure factors contains 25,969 reflections in a resolution range from 20 to 2.1 Å. The high quality of the merged data set is indicated by an R_{split} of 10.2% (which is a quality measure for SFX instead of R_{merge}) (23). Data statistics are summarized in table S2, table S3, and fig. S4. The structure was solved by molecular replacement using the coordinates of the previously determined in vitro crystallized mature TbCatB structure (Protein Data Bank ID, 3MOR) (11) as a search model.

The refined SFX TbCatB structure (R factor = 18.1%, R_{free} = 21.4%) shares the papainlike fold that is characteristic of cathepsin B-like proteases (Fig. 1C and supplementary text S1) (24), with a root mean square deviation of 0.4 Å for equivalent Cα atoms of the mature TbCatB structure determined at 100 K and refined to 2.55 Å resolution (11). The molecular replacement solution reveals electron density that is not part of the search model, which we identified as the coordinated, cleaved main part of the propeptide (residues 26 to 72) (Fig. 2A), and as two-carbohydrate

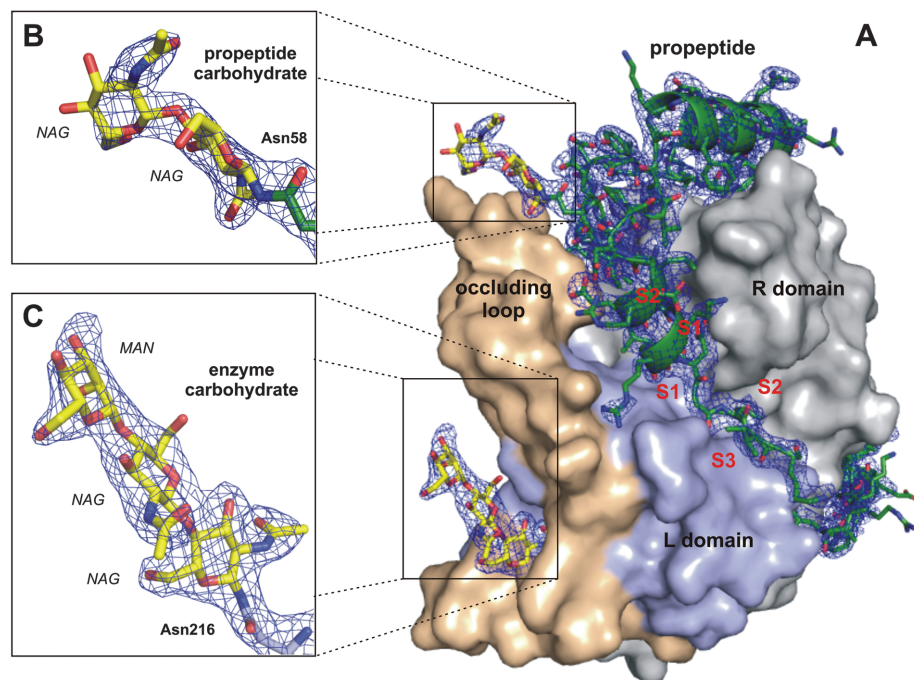


Fig. 2. Quality of the calculated electron density. (A) Surface representation of the TbCatB-propeptide complex solved by molecular replacement using the mature TbCatB structure (11) as a search model. The solution revealed additional electron density ($2F_{\text{obs}} - F_{\text{calc}}$, 1σ, blue) of the propeptide (green) that is bound to the V-shaped substrate-binding cleft and of two carbohydrate structures (yellow) N-linked to the propeptide (B) and to the mature enzyme (C). The propeptide, as well as both carbohydrates, are well-defined within the electron density map (blue), which confirms that the phases are not biased by the search model. Color codes correspond to Fig. 1C.

structures (Fig. 2, B and C). Proteolytic cleavage of the expressed precursor occurs within the propeptide between Ser⁷⁸ and Ile⁷⁹, as revealed by mass spectrometry, which leaves 15 propeptide residues bound to the N terminus of mature

TbCatB (supplementary text S2). The preceding residues Lys⁷³ to Ser⁷⁸ are disordered in the crystal structure, owing to a rise in flexibility, which shows up as a gap in the electron density in this region of the propeptide. The cleavage may be

Fig. 3. Occluding loop conformations of mature and propeptide-inhibited TbCatB. **(A)** Surface representation of mature TbCatB (11) showing the occluding loop (rigid part, beige; flexible part, red) in the closed conformation. A loop segment blocks the S2' site and part of the S1' site of the substrate-binding cleft. **(B)** Propeptide binding (green) shifts the flexible occluding loop segment (red) into an open conformation, which exposes the entire prime subsite. The insets compare the Tb (gray) and human (cyan) surface representations of mature and propeptide bound CatB. Almost superimposable Co chains of the human (blue) and Tb (beige/red) occluding loops indicate a conservation of the closed loop conformation in the mature enzymes (A), whereas the open conformations show significant differences (B). Four H bonds maintained during conformational transition restricted the flexible loop segment to four residues (red) in TbCatB, which opens a crevice of ~8.5 Å. In contrast, only one H bond is maintained in the open loop conformation of human procathepsin B (13). Thus, the mobile segment of the human occluding loop (blue) comprises 10 residues and exposes an enlarged occluding loop crevice (supplementary text S5). Residues are labeled according to TbCatB, referring to the corresponding human CatB numbering in parentheses, if applicable.

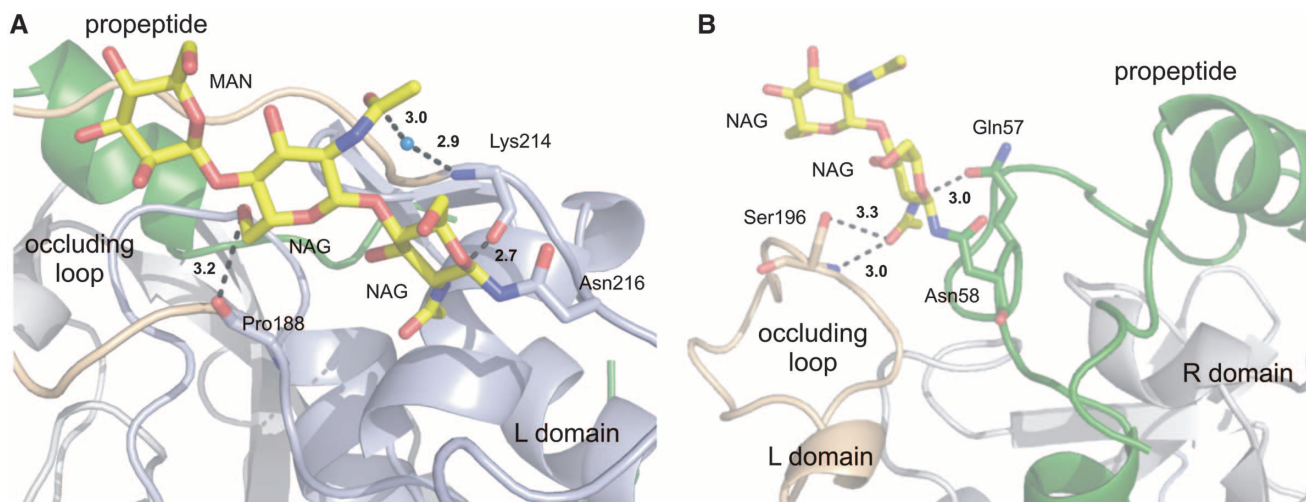
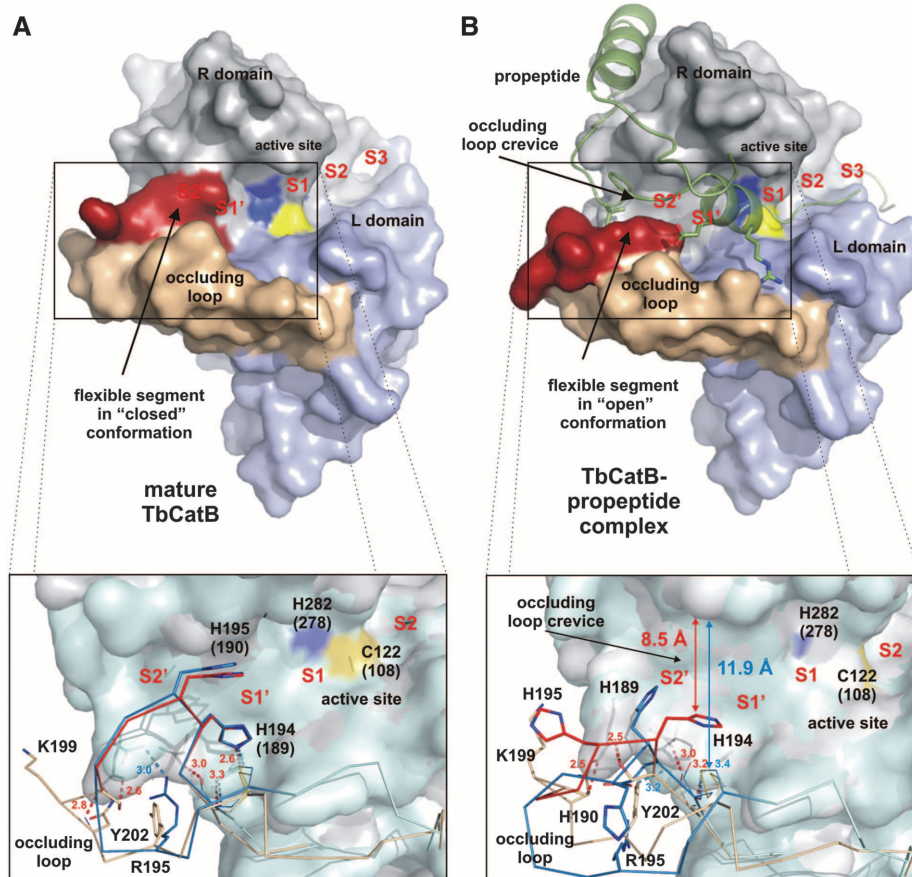


Fig. 4. Glycosylation of the TbCatB-propeptide complex. **(A)** Enzyme carbohydrate structure comprising two NAG and one MAN residue (yellow) N-linked to Asn²¹⁶ C-terminal of the occluding loop (beige). The carbohydrate structure connects both occluding loop strands by two direct and one water-bridged H bond (black dashed lines). **(B)** Propeptide glyco-

sylation site comprising two NAG units (yellow) at Asn⁵⁸ within the kinked region of the propeptide (green). The propeptide carbohydrate structure forms an H bond to Gln⁵⁷ of the propeptide and two H bonds to Ser¹⁹⁶ at the tip of the occluding loop (beige), which stabilize its open conformation. Color codes correspond to Fig. 1C.

part of the initial maturation step within the activation process of TbCatB. The final model of glycosylated TbCatB in complex with its processed, but still-bound, propeptide contains 62 propeptide residues and 247 mature enzyme residues, as well as 98 solvent and 5 carbohydrate

molecules. No electron density is observed for 11 flexible amino acid side chains or the eight atoms of the carbohydrate structures.

The SFX TbCatB structure shows that the inhibitory mechanism observed for mammalian papainlike protease-precursors remains largely

conserved in *T. brucei*, including the overall conformation of the propeptide (supplementary text S3 and fig. S5). The active site of TbCatB is blocked by the propeptide, which tightly binds in a direction the reverse of the substrate's (fig. S6) (25). A detailed comparison of the propeptide-

enzyme contact area with that observed for human procathepsin B (Protein Data Bank ID, 3PBH) (13) indicates an interface enlarged by $\sim 310 \text{ \AA}^2$ within the TbCatB-propeptide complex (supplementary text S4). Tight binding of the *T. brucei* propeptide to the enzyme interface through three conserved epitopes is maintained by 21 intermolecular polar and ionic interactions (fig. S7). These are eight fewer interactions than for human procathepsin B.

The most significant difference between the structures of mature TbCatB and the natively inhibited propeptide complex occurs in the “occluding loop” region (residues 193 to 207) (fig. S8). This highly flexible loop is a structural element characteristic of cathepsin B-like enzymes that confers exopeptidase activity (removal of dipeptide units from the C terminus of the substrate), which supplements the endopeptidase (nonterminal substrate cleavage) activity common to all papainlike proteases (26). In mature CatB, the occluding loop is in the “closed” conformation and buries an essential part of the prime subsite (S1' and S2' positions) at the substrate cleft (Fig. 3A) (27) and competes for binding with large substrates with an affinity that depends sensitively on pH (28). As a consequence of propeptide binding, the occluding loop is reoriented into an “open” conformation, exposing the entire S1' and S2' subsite of the substrate-binding cleft in TbCatB (Fig. 3B). This mirrors the open and closed conformations observed in human CatB; however, the trypanosomal occluding loop is more rigid. The displaced loop segment comprises only 4 residues rather than the 10 observed in human CatB (13). This results in a narrower exposed S2' subsite $\sim 8.5 \text{ \AA}$ wide compared with $\sim 11.9 \text{ \AA}$ for human CatB (supplementary text S5). In particular, the side chain of His¹⁹⁴ is only slightly shifted compared with the closed loop conformation and still extends into the open cleft. Thus, His¹⁹⁴ not only establishes steric constraints for the substrates but also provides a prominent polar anchor in the otherwise largely hydrophobic S2' and S1' subsites that are highly conserved between trypanosome and human CatB (fig. S9). In human CatB, the larger exposed S2' subsite in the open loop conformation is less restricted by the corresponding His¹⁸⁹ residue. This suggests that smaller hydrophobic substituents could target the prime site (S1' and S2' positions) in TbCatB, which is also supported by the propeptide structures: The bulky Phe residue that sticks into the S2' subsite of human CatB is replaced by the smaller Met of the *T. brucei* propeptide.

The occluding loop conformation is further stabilized by two carbohydrate structures identified in the TbCatB complex, as shown in Fig. 4. The enzyme carbohydrate chain interacts with

both strands of the occluding loop at the loop termini (Fig. 4A), which supports the increased loop rigidity in TbCatB mentioned above. The propeptide carbohydrate connects the tip of the open occluding loop and stabilizes the open conformation (Fig. 4B). Although N-linked oligosaccharide substitution has been detected in human procathepsin B, the predicted glycosylation sites differ from our observations in TbCatB (28, 29). Therefore, it is unlikely that the occluding loop is stabilized in a similar way in the human case (supplementary text S6). Differential glycosylation between the human and *T. brucei* precursors along with the differences in the occluding loop conformation could be exploited for synthetic parasite-specific inhibition.

As illustrated by the room-temperature glycosylated TbCatB-propeptide structure determined here, the combination of in vivo grown microcrystals with the diffraction-before-destruction technique of x-ray FELs provides a compelling path to obtain macromolecular structures from challenging samples. This methodology could vastly speed up structure determination by removing the need for large well-diffracting crystals and providing a suitable amount of crystals of posttranslationally modified proteins, in their biologically functional form.

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Supplementary Materials

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10.1126/science.1229663

CODED STORAGE TUBES

A new range of laser-etched alphanumeric coded sample storage tubes (0.5 mL, 0.75 mL, 1.1 mL, and 1.4 mL) is now available. The new tubes have a flat polypropylene bottom on which the alphanumeric code is encrypted with a laser. Uniquely manufactured from a single component—the laser-encrypted alphanumeric codes cannot fade or peel off even when subjected to repeated handling, exposure to different solvents, or multiple freeze-thaw cycles. Because code clarity will remain unchanged throughout their lifetime, the laser etched alphanumeric coded tubes offer the security of unmatched sample traceability. Manufactured from high-quality polypropylene in a fully automated class-7 cleanroom environment ensures the laser-etched alphanumeric tubes exhibit absolute product consistency, near-zero contaminants, and compliance with U.S. and European Pharmacopoeia tests. The laser-etched alphanumeric tubes resist many organic solvents (DMSO, methanol, dichloromethane), may be autoclaved clean, and can be repeatedly freeze-thawed without loss of product performance.

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CIRCULATING BATH

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ELECTROPORATION SYSTEM

Developed to meet the growing need for in-situ transfection of hard-to-transfect adherent cell types, the new Cellaxess ACE Adherent Cell Electroporation System enables transfection and delivery of nongenetic material in any adherent cell type at any developmental stage. The platform enables in situ primary and iPSC-derived cell types directly in 96- and 384-well microplates and cell culture dishes at any cellular developmental stage with excellent viability and completely retained cellular morphology. Cellaxess ACE is available as a complete system, but can also be integrated with third-party pulse generators. Applications for the Cellaxess ACE include transfection of various types of neuronal cells such as hippocampal and cortical neurons, dorsal root and superior cervical ganglia from rat or mouse, and human iPSC-derived dopaminergic neurons. Protocols are available for a wide range of neuronal cells as well as other adherent cell types.

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CELL FERMENTATION PROCESS ANALYTICS

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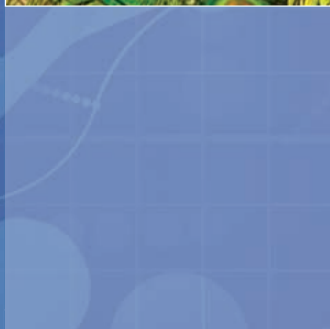
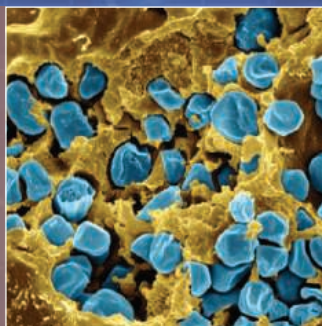
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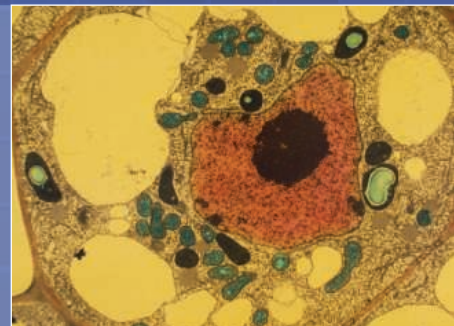
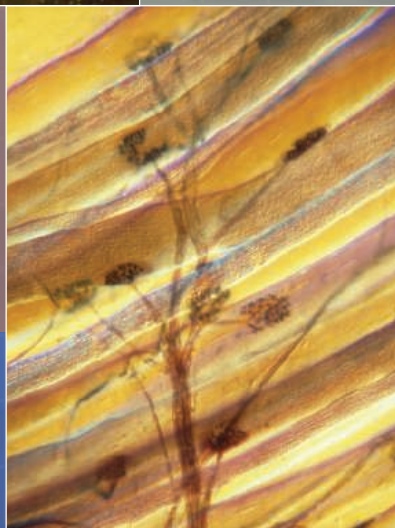
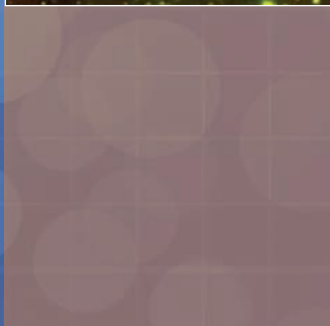
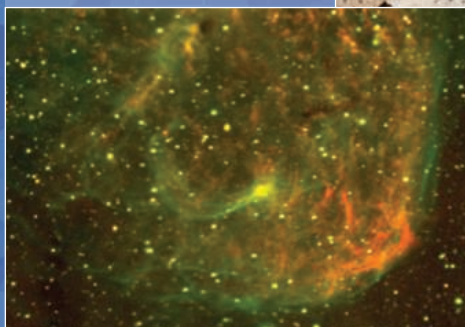
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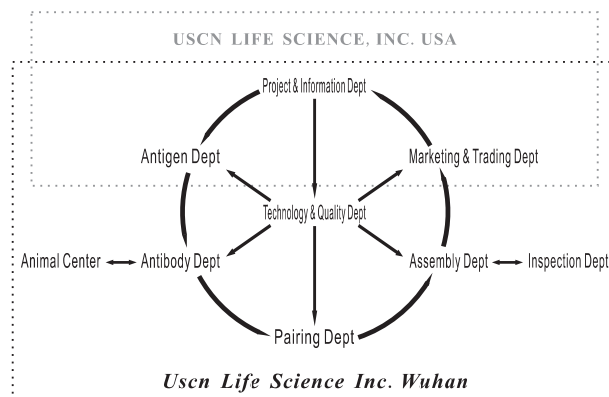


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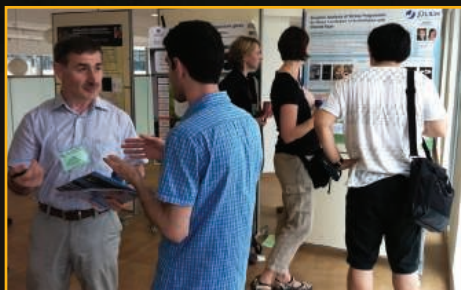
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Chair: Wai-Yee Chan

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Chair: Roberto Bruzzone

Marine Molecular Ecology

August 11-16, 2013

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and Technology

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Chairs: Pei-Yuan Qian &

Roberto G. Kolter

Nano-Mechanical Interfaces

Multiphysics Theory and Experiments

August 4-9, 2013

Hong Kong University of Science

and Technology

Hong Kong, China

Chair: Alfonso Ngan

Posttranslational Modification Networks

Phosphosignaling

July 28 - August 2, 2013

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Chair: Ning Li

Spin Dynamics in Nanostructures

August 18-23, 2013

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Chair: Xiang Rong Wang

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July 21-26, 2013

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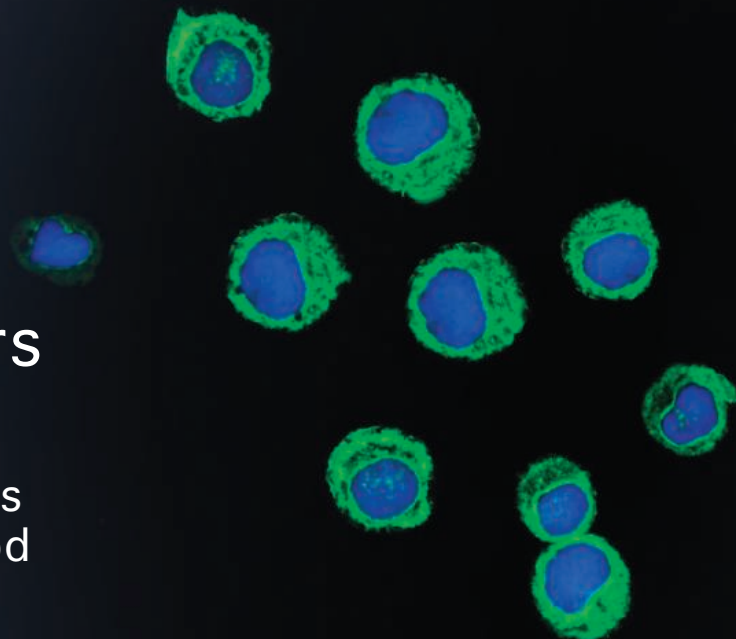
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WEBINAR

Genetic Biomarkers Revealed:

Unraveling the Complexities of Cancer Genomes in Blood Malignancies



WEDNESDAY, JANUARY 30, 2013

11 a.m. ET, 8 a.m. PT, 4 p.m. GMT, 5 p.m. CEST

SPEAKERS

Detlef Haase, M.D., Ph.D.
University of Göttingen,
Germany

Stuart Schwartz, Ph.D.
Labcorp
Research Triangle Park, NC

Hematological or blood malignancies are types of cancer that affect blood, bone marrow, and lymph nodes, such as leukemias and lymphomas. To truly understand the complexities of their biology requires a combination of genomic, epigenomic, and functional analysis. In the past decade, research has increasingly shown that DNA copy number changes and rearranged chromosomal regions are associated with cancer susceptibility. Identifying these cytogenetic biomarkers is key to understanding clonal identities, evolution, response to treatment and relapse. Improving the understanding of these genetic changes in these diseases, can point to new directions for diagnosis and treatment, including a way to potentially differentiate aggressive tumors from those that are not life threatening. Our expert panel will describe their research and the discoveries they have made that are increasing the understanding of the genetic basis of hematological malignancies.

DURING THE WEBINAR, THE SPEAKERS WILL:

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- Discuss the potential implications of their findings in terms of diagnosing, classifying, and treating patients in the future.
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UC San Diego
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Dean and Associate VC for Health Sciences

Job Number: 10-521

Hiring Unit: Health Sciences

Salary: Salary is negotiable and commensurate with qualifications and experience and based on UC pay scales

Closing Date: Confidential review of applications and nominations will begin February 15, 2013 and will continue until an appointment is made.

UNIVERSITY OF CALIFORNIA, SAN DIEGO –

DEAN, SKAGGS SCHOOL OF PHARMACY AND PHARMACEUTICAL SCIENCES

The University of California, San Diego (UC San Diego) Health Sciences is committed to academic excellence and diversity within the faculty, staff, and student body and invites applications for the dual position of Dean of its Skaggs School of Pharmacy and Pharmaceutical Sciences (SSPPS) and Associate Vice Chancellor for Health Sciences. Having matriculated its first students in 2002, SSPPS has gained international prominence as one of the leading pharmacy schools in a research-intensive university. Based on the La Jolla campus of UC San Diego, SSPPS interacts closely with UC San Diego entities (the School of Medicine, the Rady School of Management, the Graduate School, the Scripps Institution of Oceanography, the Division of Physical Sciences, the Division of Biological Sciences, and the Health System), the Rady Children's Hospital of San Diego, the Veterans Affairs San Diego Healthcare System, the J. Craig Venter Institute, the La Jolla Institute of Allergy and Immunology, the Salk Institute, the Scripps Research Institute, the Sanford-Burnham Medical Research Institute, and the Sanford Consortium for Regenerative Medicine. It is the only school of pharmacy in the region.

Reporting to the Vice Chancellor for Health Sciences, the Dean and Associate Vice Chancellor will provide strategic vision and operational leadership for the school. The Dean will advance scholarship, education, research, and outreach. The school has 42 salaried fulltime faculty, 250 voluntary faculty, 240 Doctor of Pharmacy students, 50 graduate students, and 30 pharmacy residents. The SSPPS also features a dual Doctor of Pharmacy/Doctor of Philosophy program and is developing additional combined degree programs. A novel joint curriculum with the UC San Diego School of Medicine is a hallmark of the Doctor of Pharmacy program. The school attracts more than \$10 million of extramural funding per year.

The successful candidate will be an internationally recognized leader in pharmacy with an outstanding academic record, and documented administrative experience (e.g., current dean, department chair, program director). Minimum requirements include a Doctor of Pharmacy and/or Doctor of Philosophy degree or an equivalent professional degree or experience; a strong commitment to research, teaching, clinical service, and public service; and a personal record of distinguished scholarly and/or clinical accomplishment and the ability to qualify for appointment as a full professor in an appropriate University of California academic series.

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Dean and Associate VC for Health Sciences

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FACULTY POSITIONS: CHEMICAL SCIENCE

The Chemical Science Program in the Physical Sciences and Engineering Division at King Abdullah University of Science and Technology (KAUST) invites applications for faculty positions at all ranks (Assistant, Associate, and Full Professors).

King Abdullah University of Science and Technology (KAUST) is an international, graduate research university dedicated to advancing science and technology through interdisciplinary research, education, and innovation. Located on the shores of the Red Sea in Saudi Arabia, KAUST offers superb research facilities, generous assured research funding, and internationally competitive salaries. The university attracts top international faculty, scientists, engineers, and students to conduct fundamental and goal-oriented research to address the world's pressing scientific and technological challenges related to the sustainability of water, food, energy, and the environment.

The Physical Sciences and Engineering Division (PSE) encompasses five core disciplines: Chemical and Biological Engineering; Chemical Science; Earth Science and Engineering; Materials Science and Engineering; and Mechanical Engineering. The science produced in PSE is about understanding, modeling, and manipulating matter at all scales: nano, meso, and macroscopic levels; in all forms: bulk, thin films, divided colloids, fluid flows, earth as system etc.; and in interaction with external stimuli: light, heat, fluids, etc.; or stresses. The knowledge created serves to design and engineer materials, technologies, and systems. Find more information about PSE at <http://pse.kaust.edu.sa>.

The Chemical Sciences Program is based on Chemistry as a core discipline, yet it promotes interactions between KAUST Research Centers, particularly the Catalysis, Membrane, Solar Energy, Red Sea Sciences, and Geometric Modeling and Visualization Centers. Research opportunities in the Chemical Science Program are related to KAUST's strategic goals targeting future needs of the global community. Additional information is available at: <http://me.kaust.edu.sa/>

The Chemical Science program is currently recruiting for the following faculty positions:

- Polymeric Materials:

With emphasis on the design, synthesis, characterization (structural and molecular), and properties of novel well-defined polymeric materials in line with KAUST's focus in the areas of water, food, energy, and the environment.

- Experimental Polymer Physics:

With emphasis on the dynamics and molecular rheology of polymeric systems including, but not limited, branched polymers, copolymers, functionalized and responsive polymers, nanocomposites, melts, and solutions. Particular interest will be given to applicants with research experience and related background in polymer processing.

- Total Synthesis:

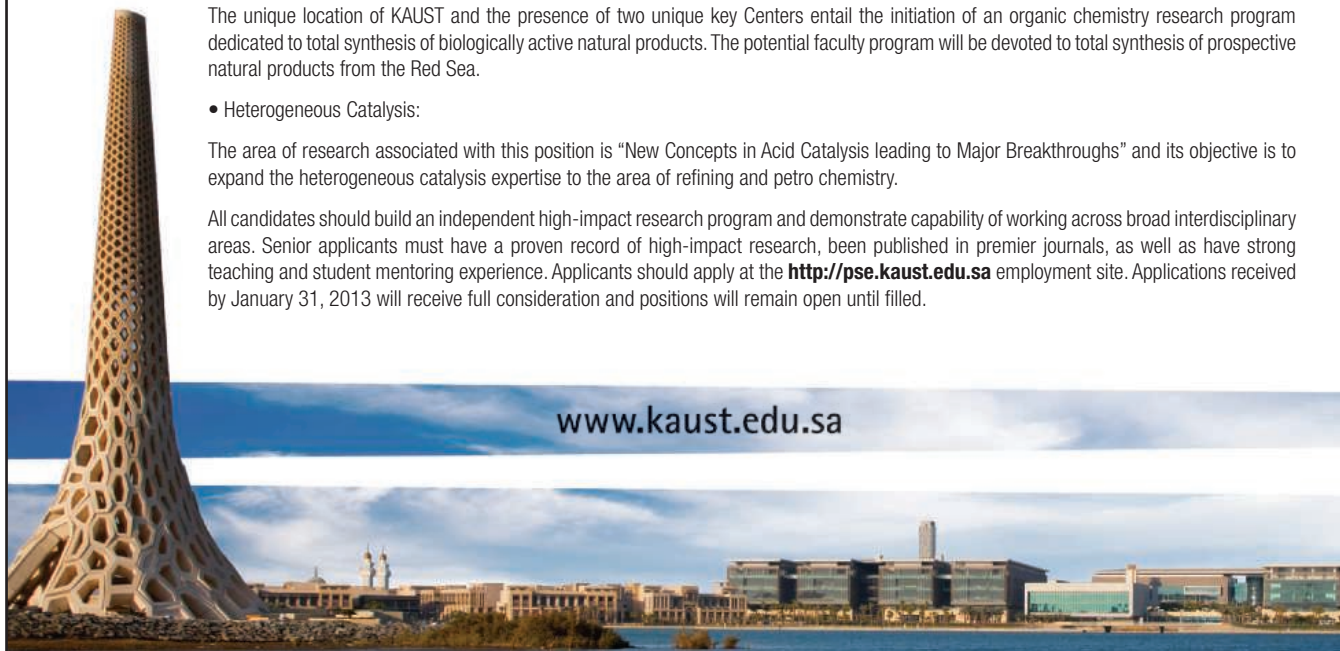
The unique location of KAUST and the presence of two unique key Centers entail the initiation of an organic chemistry research program dedicated to total synthesis of biologically active natural products. The potential faculty program will be devoted to total synthesis of prospective natural products from the Red Sea.

- Heterogeneous Catalysis:

The area of research associated with this position is "New Concepts in Acid Catalysis leading to Major Breakthroughs" and its objective is to expand the heterogeneous catalysis expertise to the area of refining and petro chemistry.

All candidates should build an independent high-impact research program and demonstrate capability of working across broad interdisciplinary areas. Senior applicants must have a proven record of high-impact research, been published in premier journals, as well as have strong teaching and student mentoring experience. Applicants should apply at the <http://pse.kaust.edu.sa> employment site. Applications received by January 31, 2013 will receive full consideration and positions will remain open until filled.

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The applicants will be expected to maintain a vigorous research program and participate in teaching and service for IMM. The successful candidate will possess a PhD and/or MD degree and also is fluent in both English and Chinese. Applicants should submit (1) curriculum vitae, (2) a description of research accomplishments and (3) an email title with the position intended to apply electronically to jdlee@scripps.edu, jdleeszu@gmail.com and jdlee@szu.edu.cn. Review of applications will begin immediately, and continue until the positions are filled. Details please see Job Ad in Shenzhen University Website (<http://szuhr.szu.edu.cn/>).



University of Michigan
Geriatrics Center

Biology of Aging

The University of Michigan Geriatrics Center is seeking to hire a tenure track faculty member to conduct biogerontology research. The ideal candidate would be working on a fundamental problem in the cellular and molecular biology of aging, using a mixture of genetic, bioinformatics, and biochemical methods, and committed to establishing a reputation as a leader in aging research. Successful candidates will be housed in modern, dedicated Biogerontology lab space, and will receive a primary appointment in a basic science or research-oriented clinical department as appropriate. Minimum qualifications are a PhD, MD, or equivalent, several years of highly productive postdoctoral research, and a clear interest in problems relevant to aging. The Geriatrics Center provides a superb environment for research on aging, including NIA support for its Nathan Shock Center, Claude Pepper Center, and Aging Training programs. Substantial start-up funding will be available.

Applicants should submit a CV, a 1-2 page description of research interests, a synopsis of current and previous research support, and contact information for 3 – 5 referees, to **Rich Miller, 3001 BSRB, Box 2200, 109 Zina Pitcher Place, Ann Arbor, MI 48109-2200**; or by e-mail to millerr@umich.edu.

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A globally connected cosmopolitan city, Singapore provides a supportive environment for a vibrant research culture. Its universities Nanyang Technological University (NTU), National University of Singapore (NUS) and Singapore University of Technology and Design (SUTD) invite outstanding young researchers to apply for the prestigious TRF awards.

Under the TRF scheme, selected young researchers with a PhD degree have an opportunity to conduct and lead defence-related research. It offers:

- A 3-year research grant of up to S\$1 million commensurate with the scope of work, with an option to extend for another 3 years
- Postdoctoral or tenure-track appointment (eligibility for tenure-track will be determined by the university)
- Attractive and competitive remuneration

Fellows may lead, conduct research and publish in these areas:

- Advanced Materials for Aerospace Applications
- Bio-mimetic Aerodynamics
- Cognitive Science and Neuroengineering
- Cyber Security
- High Power Laser Diode
- High Speed High Voltage Switching Devices

For more information and application procedure, please visit:

NTU – http://www3.ntu.edu.sg/trf/index_trf.html
NUS – <http://www.nus.edu.sg/dpr/funding/trf.html>
SUTD – <http://www.sutd.edu.sg/trf>

Closing date: 15 March 2013 (Friday)

Shortlisted candidates will be invited to Singapore to present their research plans, meet local researchers and identify potential collaborators in July 2013.

Duke

NICHOLAS SCHOOL OF THE
ENVIRONMENT



Faculty of Excellence Initiative

The Nicholas School of the Environment at Duke University seeks outstanding scholars with exceptional records or promise of interdisciplinary research, productivity, creativity, vision, and leadership.

The mission of the Nicholas School of the Environment is to create knowledge and leaders of consequence for a sustainable future. Our focus is on leadership in education, research, and service to understand environmental processes, to understand human behavior related to the environment, and to inform society about the conservation and enhancement of our environment and natural resources for future generations. In keeping with our interdisciplinary culture, we seek individuals with exceptional promise or demonstrated leadership whose work spans disciplinary boundaries (earth system science, environmental life sciences, environmental social sciences) on the general themes of **marine science and governance, environmental health, biodiversity and ecosystem services, and energy and the environment**. Applications are strongly encouraged from members of under-represented populations.

Appointments may be made at the Assistant, Associate, or Full Professor level, and may be based at either Duke's Durham NC campus or at the Duke Marine Laboratory in Beaufort NC. We seek candidates with a demonstrated record or exceptional promise of achievement, and leadership in their fields of expertise. Candidates will be expected to teach and mentor students at the undergraduate level, professional master and/or doctoral levels, and to support a vigorous, extramurally funded research program.

Please send a statement of interest, including a summary of research and teaching, a curriculum vitae, and contact information for three referees as a single PDF file to: **Laura Turcotte, lturcotte@duke.edu**

Applications will be considered as they are received and the search will remain open until positions are filled.

Duke University is an Affirmative Action/ Equal Opportunity Employer.

FACULTY POSITIONS: CHEMICAL AND BIOLOGICAL ENGINEERING

The Chemical and Biological Engineering Program in the Physical Sciences and Engineering Division at King Abdullah University of Science and Technology (KAUST) invites applications for faculty positions at all ranks (Assistant, Associate, and Full Professors).

King Abdullah University of Science and Technology (KAUST) is an international, graduate research university dedicated to advancing science and technology through interdisciplinary research, education, and innovation. Located on the shores of the Red Sea in Saudi Arabia, KAUST offers superb research facilities, generous assured research funding, and internationally competitive salaries. The university attracts top international faculty, scientists, engineers, and students to conduct fundamental and goal-oriented research to address the world's pressing scientific and technological challenges related to the sustainability of water, food, energy, and the environment.

The Physical Sciences and Engineering Division (PSE) encompasses five core disciplines: Chemical and Biological Engineering; Chemical Science; Earth Science and Engineering; Materials Science and Engineering; and Mechanical Engineering. The science produced in PSE is about understanding, modeling, and manipulating matter at all scales: nano, meso, and macroscopic levels; in all forms: bulk, thin films, divided colloids, fluid flows, earth as system etc.; and in interaction with external stimuli: light, heat, fluids, or stresses. Find more information about PSE at <http://pse.kaust.edu.sa>.

The Chemical and Biological Engineering Program offers opportunities to develop real-world solutions to global challenges. These include the development of new membranes for gas and liquid separations, as well as new materials for reducing greenhouse gases and remediating chemical and biological threats. Alternative and renewable energy processes, and new methods for carbon dioxide utilization all contribute to the overall research mission of KAUST.

The Chemical and Biological Engineering program is currently recruiting for the following positions:

- Process Modeling and Design

The candidate should be able to conduct design, optimization, and cost analysis of membrane and conventional separation processes and conduct teaching of advanced principles of process design and control.

- Petroleum and Natural Gas Engineering

The candidate must have strong knowledge of the petroleum and/or natural gas industry and build a bridge between KAUST and Saudi Arabia's fast growing petroleum industry.

- Biomolecular Engineering

The candidate should have a well-established research career in bioprocess engineering or biomedical engineering.

- Nanotechnology for Sustainable Energy

A strong research background in areas such as development of nanostructured materials and processes for energy conversion or catalysis with improved efficiency and/or reduced cost is required.

All candidates should build an independent high-impact research program and demonstrate capability of working across broad interdisciplinary areas. Senior applicants must have a proven record of high-impact research, been published in premier journals, as well as have strong teaching and student mentoring experience.

Applicants should apply at the <http://pse.kaust.edu.sa> employment site. Applications received by January 31, 2013 will receive full consideration and positions will remain open until filled.

www.kaust.edu.sa



Assistant Professor – Medicinal Chemistry

The Department of Pharmacology and Toxicology, in the College of Pharmacy, at the University of Arizona, invites applications for two tenure track or tenured faculty positions at the Assistant Professor rank. We are seeking outstanding medicinal chemists to join our expanding program in the newly formed Drug Discovery Center of Excellence at Bio5 Oro Valley. Candidates should have a Ph.D. in pharmacology, medicinal chemistry, toxicology, or related discipline, and must provide evidence of, or potential to develop a rigorous and independent research program. Applicants with expertise in chemical-biology, natural-products based drug discovery, computer-based drug design, high-throughput assay development and/or with experience of advancing small molecules along the drug discovery value chain in a high-throughput manner are encouraged to apply. The Drug Discovery and Development program is undergoing a period of dynamic growth in parallel with significant facilities expansion. Excellent opportunities also exist for research collaborations and participation in numerous Centers of Excellence that include the Arizona Cancer Center, Bio5 Institute, SWEHSC, Sarver Heart Center, and Arizona Respiratory Center. Successful candidates will participate in graduate (Ph.D.) and professional (Pharm.D.) education.

For complete details and to apply see **job 51301** at **www.uacareertrack.com**. Review of applications will begin **January 11, 2013** and continue until filled.

Salary: Depending on Experience - plus outstanding UA benefits: health, dental and life insurance; paid vacation, sick leave and holidays; UA/ASU/NAU tuition reduction for employee and qualified family; access to campus cultural and recreational activities; state retirement and more.

The University of Arizona is an EEO/AA Employer-M/W/D/V.



Assistant/Associate Professor – Pharmacology/Toxicology

The Department of Pharmacology and Toxicology, in the College of Pharmacy, at the University of Arizona, invites applications for a tenure track or tenured faculty position at the Assistant or Associate Professor rank. Candidates should have a Ph.D. in pharmacology, toxicology, or related discipline, or M.D., and must provide evidence of, or potential to develop a rigorous and independent research program suitable for graduate student research training. Individuals with research interests broadly in personalized medicine (drug metabolism and disposition, epigenetics) or complementary to established departmental strengths (cell signaling/stress response, chemoprevention, environmental health) are preferred. It is anticipated that successful candidates will contribute to the Southwest Environmental Health Sciences Center (SWEHSC) and participate in graduate (Ph.D.) and professional (Pharm.D.) education. Excellent opportunities exist for research collaborations and participation in numerous Centers of Excellence that include the Arizona Cancer Center, Bio5 Institute, SWEHSC, Sarver Heart Center, and Arizona Respiratory Center.

For complete details and to apply see **job 51300** at **www.uacareertrack.com**. Review of applications will begin **January 11, 2013** and continue until filled.

Salary: Depending on Experience - plus outstanding UA benefits: health, dental and life insurance; paid vacation, sick leave and holidays; UA/ASU/NAU tuition reduction for employee and qualified family; access to campus cultural and recreational activities; state retirement and more.

The University of Arizona is an EEO/AA Employer-M/W/D/V.

Duke University Pratt School of Engineering

Faculty Position in Civil and Environmental Engineering

The Department of Civil and Environmental Engineering in the Pratt School of Engineering at Duke University is seeking an extraordinarily qualified candidate for a faculty position at the level of Tenure-track Assistant Professor. The thrusts of the Department include environmental nanotechnologies and fluid mechanics, ecohydrology, hydrometeorology and water resources and sustainability. Candidates working on the areas pertaining to (i) interactions between the variability in the hydrological cycle and climate, (ii) the consequences of such interactions for water resources, energy and ecosystems, and (iii) the development of novel engineering solutions for adapting to their effects on society and in particular the water-energy nexus are especially encouraged to apply. The successful candidate is expected to be involved in Duke's broader efforts in water resources, hydrology, climate, ecology, and related interdisciplinary areas. Opportunities to collaborate in cross-disciplinary initiatives with the Center on Global Change, Center for Theoretical and Mathematical Sciences, Center for Nonlinear and Complex Systems, Duke Global Health Institute, the Nicholas Institute on Environmental Policy Solutions, the Nicholas School of the Environment, and the Sanford School of Public Policy are available. Qualified candidates must have a Ph.D. in engineering or related physical sciences.

Letters of application (including names and contact information of at least three potential references, curriculum vitae, description of research and teaching interests as a single pdf file) should be submitted attached to an email to (**cee-faculty-search@duke.edu**) with subject 'CEE hydrology search'. The application review process will commence on **January 15, 2013**, and will continue until the position is filled.

Duke University is an Affirmative Action Equal Opportunity Employer that is committed to increasing the diversity of its faculty.

Science Careers

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For your career in science, AAAS
there's only one **Science**

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Career advice | Job postings | Job Alerts | Career Forum
Crafting resumes/CVs | Preparing for interviews

Call for Applications for the Directorship of IBS Research Centers

The Institute for Basic Science (IBS) was established by the Korean government in November 2011 with the goal to create a world-class research institute in the basic sciences. To achieve the goal, IBS has appointed 16 internationally renowned scholars as IBS Director and is going to appoint 34 more directors until 2017.

Now, IBS invites applications for the IBS Directorship in the designated research area. The Designated Research Area was selected to foster the basic science which is of national and international importance for the future and to create research hub for each research area. The designated research areas and specific research contents are as follows:

- **Mathematics:** Scientific Computing, Arithmetic and Algebraic Structure, Randomness, Nonlinearity, Dynamics
- **Theoretical Fundamental Physics:** Particle & Nuclear Theory, String Theory & Gravitation/Field Theory, Astrophysics & Cosmology, Other related research areas
- **Condensed-Matter and Complex Systems Theory:** Strongly Correlated Electron System, Statistical Physics, Biological Physics, Computational Physics, Interdisciplinary Physics Studies, Other related research areas
- **Science of Global and Regional Environmental Changes:** Anthropogenic Climate Forcing and Biogeochemical Cycles, Climate Physics, Responses of Marine Ecosystem, The Coastal Zone including Extreme Events, Integrated Impact Assessment of above, Other related research areas
- **Optics-based Basic Science:** Ultraintense Field Optics, Photochemistry, Optical Spectroscopy, Photobiophysics, Other related research areas
- **Basic Science for Advanced New Materials:** New Carbon Allotropes, Molecule-based Bio-materials, Materials for Sustainable Energy, Other related research areas
- **Basic Science for Bio-Nano Convergence:** Bioinspired materials, Functional Regulators of Cellular Organelles, Novel Magnetic Materials for Nanomedicine and Biosensing, Plant Biomaterials, Other related research areas

Additional Information: Please visit our website at <http://www.ibs.re.kr/en> to see the full information on the invitation and to see the frequently asked questions (FAQ) on IBS Research Center.

Application Submission: Please download application form and send your application as a single PDF to: <http://www.ibs.re.kr/en/apply>. Applications in the designated research area listed above are open for two years from the date of the announcement; applications submitted before **31st January, 2013** will be reviewed first.

Inquiries: (Tel) +82-42-878-8172,
(e-mail) evaluation@ibs.re.kr

Note: Application including modified research area within IBS designated research area may be accepted.

TUM is the first university in Germany to reinforce its recruitment policy with a comprehensive tenure track system. Based on best international standards and transparent performance criteria, **TUM FACULTY TENURE TRACK** offers merit-based academic career options for high-potential young scientists, from the appointment as Assistant Professor through a permanent position as Associate Professor and on to Full Professor.

The **Institute for Advanced Study of the Technische Universität München (TUM-IAS)** invites applications for the prestigious

Rudolf Mößbauer Tenure Track Assistant Professorship

initially pay-scale grade W2.

Within its Fellowship program, the TUM-IAS awards up to four Rudolf Mößbauer Tenure Track Professorships to outstanding, high-potential young scientists who have already achieved a major scientific or technological breakthrough and who have the ambition of developing a new field of endeavor at TUM. Applications are invited in the following areas of the TUM research portfolio:

- Biomedical and Clinical Instrumentation
- Bioinorganic Chemistry, Surface Organometallic Catalysis and Electrocatalysis
- Molecular and Cellular Engineering
- Theoretical Foundations of Technology
- Nanoscience
- Environmental and Energy Engineering
- Systems Modeling, Knowledge Engineering and Computing

The initial appointment will be for six years. After positive evaluation in the sixth year, the candidate is tenured on an Associate Professor level. In exceptional cases, the tenure evaluation may be initiated after a minimum of three years. Such cases will have to be justified by outstanding achievements of the candidate and when the candidate contributes to strategically shaping the university's profile.

Eligible candidates hold a doctorate, have established a strong track record in the postdoctoral phase, can show evidence of at least one major scientific contribution, and have demonstrated pedagogical and personal aptitude. Candidates must also have considerable international experience and fulfill the mobility requirements of the Marie Curie COFUND program within EU's 7th Framework Program (i.e., no more than 12 months residence and/or employment in Germany in the three years preceding the publication date of the call).

More information on TUM-IAS, the Rudolf Mößbauer Tenure Track Professorship, and detailed employment conditions can be found under: www.tum-ias.de/how-to-apply/rudolf-moessbauer-tenure-track.html

Rudolf Mößbauer Tenure Track Fellows are expected to develop independent and vigorous programs, including the acquisition of research funds and the societal positioning of their fields. Furthermore, candidates should be committed to excellence in undergraduate/graduate teaching and in supervising PhD students, although the emphasis of the Professorship lies in the creative development of the proposed new field of science and/or technology.

As part of the Excellence Initiative of the German federal and state governments, TUM is pursuing the strategic goal of substantially increasing the diversity of its faculty. As an equal opportunity and affirmative action employer, TUM explicitly encourages nominations of and applications from women as well as from all others who would bring additional diversity dimensions to the university's research and teaching strategies. Preference will be given to disabled candidates with essentially the same qualifications. The TUM Munich Dual Career Office provides support for dual-career couples and families.

Applications are submitted online via www.onlineforms.tum.de/rmttf/ and should include the following documents: CV, list of publications including three selected reprints, a statement on research and teaching goals and strategies, and the names and addresses of at least three references. The deadline for application is **February 28, 2013**.

**Institute for Advanced Study
Technische Universität München
Garching, Germany**

Inquiries should be addressed to:
info@tum-ias.de

Science

Innovations & Opportunities: **INDIA**

As the second-most populous country with more than 1.2 billion people, India is positioning itself to become a leader in scientific research. *Science* will take an in-depth look at research innovation and career opportunities within the country.



Content: Our February 22 special advertising feature will examine the Indian government's recent infusion of support for scientists and researchers. The feature will also explore the resulting innovations. This renewed commitment to growth creates opportunities both for technology development and service suppliers, as well as for job seekers.

Reach: 700,000 scientists from around the world will read about these exciting developments in India. Whether your goal is recruitment or promoting your products and services, this issue offers a unique chance to reach a global scientific community that all share a common interest in being a part of India's growth opportunities.

Results: *Science* offers a simple formula: relevant content that spotlights your ad + a large, qualified audience = your hiring success.

Reach more scientists by advertising in this special feature.

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SPECIAL FEATURE:

February 22, 2013

Reserve your ad by February 5 to guarantee space.*

*Ads accepted until February 15 if space is still available.

To book your ad, contact Lucy Nelson

Phone: +44 (0)1223 326527

E-mail: lnelson@central.science-int.co.uk



Professor of Geoenergy Process Technologies

The Department of Mechanical and Process Engineering (www.mavt.ethz.ch) at ETH Zurich invites applications for a full professorship or an assistant professorship (tenure track) in Geoenergy Process Technologies.

Applicants should demonstrate a strong background in engineering and natural sciences and, in particular, geophysics, geochemistry or systems thermodynamics. They should have a strong motivation and undisputable commitment to undergraduate and graduate student education. The successful candidate is expected to establish an ambitious, world-class program in a research-intensive crossdisciplinary environment of the department, where state-of-the-art characterization, analysis, and synthesis research are ongoing. A close cooperation with the Department of Earth Sciences (www.erdw.ethz.ch) is expected. Excellent research and teaching lab facilities are being established across ETH Zurich. Furthermore, the Swiss research and industrial landscape offers extraordinary opportunities, particularly in energy technology.

Candidates should have a PhD degree in process engineering, chemical engineering, geoengineering or a related field and have an excellent international record of accomplishments. The successful candidate will be expected to teach undergraduate level courses (German or English) as well as graduate level courses (English).

Applications should include a curriculum vitae, a list of major achievements and a list of refereed publications. The letter of application should be addressed **to the President of ETH Zurich, Prof. Dr. Ralph Eichler. The closing date for applications is 31 March 2013.** ETH Zurich is an equal opportunity and affirmative action employer. In order to increase the number of women in leading academic positions, we specifically encourage women to apply. ETH Zurich is further responsive to the needs of dual career couples and qualifies as a family friendly employer. **Please apply online at www.facultyaffairs.ethz.ch.**

Professor of Deep Geothermal Energy and Geological Reservoirs

The Department of Earth Sciences (www.erdw.ethz.ch) at ETH Zurich invites applications for a professorship in Geothermal Reservoir Engineering, covering deep geothermal energy and related processes in geological reservoirs. The professorship is opened at the full professor level, but exceptional young scientists at the tenure track assistant professor level are also encouraged to apply.

The new professor will build a vibrant research group, focussed on understanding the relevant geoscientific processes of deep reservoirs in the Earth's crust, and will contribute to an undergraduate (German or English), a graduate (English) as well as a practice-oriented teaching program in this field. Research will address interacting geological, physical and chemical processes from the short timescale of rock fracturing to the longer time scale of fluid flow and heat production over the lifetime of an engineered geological reservoir. Key disciplines include fractured reservoir hydraulics, geomechanics and reservoir engineering with a strong link to earth sciences. Further interests in other energy-relevant uses of geological reservoirs are welcome, e.g. in CO₂ storage, coal-bed methane generation and conventional and unconventional oil and gas reservoirs.

The successful candidate will have a strong academic track record and geoscientists with a current private-sector or academic background are equally encouraged to apply. The new professor will foster links between fundamental geoscience research and application by government and industrial stakeholders, and is expected to take a leading role in pilot/demonstration projects under development in Switzerland and worldwide. He/she will cooperate with existing professorships in Earth Sciences (including engineering geology, structural geology, geochemistry, seismology, applied and exploration geophysics), and with a new professorship focussed on engineering aspects of geothermal energy use, to be established at the Department of Mechanical and Process Engineering.

Applications should include a curriculum vitae, a list of publications and a statement of research and teaching interests. The letter of application should be addressed **to the President of ETH Zurich, Prof. Dr. Ralph Eichler. The closing date for applications is 31 March 2013.** ETH Zurich is an equal opportunity and affirmative action employer. In order to increase the number of women in leading academic positions, we specifically encourage women to apply. ETH Zurich is further responsive to the needs of dual career couples and qualifies as a family friendly employer. **Please apply online at www.facultyaffairs.ethz.ch.**



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To learn more, visit aaas.org/plusyou/sciencecareers



Announcing a New U.S./Australian Fellowship Program in Plant Biosecurity

Applications are now being accepted for six doctoral Fellowships to conduct collaborative research in plant biosecurity. Fellowship recipients will enroll in one of K-State's renowned departments of Plant Pathology, Grain Science or Entomology or the interdepartmental Genetics program, and conduct collaborative research through the Australian Plant Biosecurity Cooperative Research Centre. The Fellowship award includes annual travel to Australia for research and professional development.

Apply immediately through the Kansas State University
Graduate School at k-state.edu/grad/crc

Application materials due Feb. 15.

Additional doctoral Fellowships are also available in genetics!
Visit <http://www.ksre.k-state.edu/genetics/>

KANSAS STATE
UNIVERSITY



Cleveland Clinic

VISION RESEARCH SCIENTISTS Cole Eye Institute The Cleveland Clinic

In conjunction with a major expansion of the Cole Eye Institute, applications are being accepted for full-time research positions at the Assistant, Associate or Full Professor levels. Basic and clinician scientist with training in molecular genetics, molecular biology, cell biology, oncology, pharmacology, physiology, biophysics, biochemistry, developmental biology or gene therapy with research interests that are focused on understanding basic mechanisms related to corneal disease, glaucoma, macular degeneration, or inherited eye disorders will be seriously considered. Excellent laboratory space, generous startup funds, outstanding salary support and benefits package are included. Applicants for positions at Associate and Full Professor level must have a solid record of independent funding with ongoing grant support.

Interested applicants should submit a statement of their research interests related to one or more the above areas, a copy of their curriculum vitae, and contact information for three references to:

Joe G. Hollyfield, Ph.D.
Chairman, Ophthalmic Research
Cole Eye Institute (i31)
Cleveland Clinic
9500 Euclid Avenue
Cleveland, Ohio 44195-5245
Fax: (216) 445-3670
E-mail: hollyfj@ccf.org

CCF is an Equal Opportunity/Affirmative Action Employer.

Core-funded Junior Group Leader Positions



Paterson
Institute for Cancer Research

- 4 vacancies
- Job Reference number: PI/13/01

The Paterson Institute for Cancer Research (www.paterson.man.ac.uk) is seeking four tenure-track Junior Group Leaders to join a vibrant programme of basic, translational and clinical research. The Paterson Institute is one of Europe's premier cancer research centres and is core-funded by Cancer Research UK, the world's largest independent cancer research charity. It is an Institute of the University of Manchester and lies at the heart of the Manchester Cancer Research Centre (MCRC), an exciting development that integrates cancer research across Manchester (www.mcrc.manchester.ac.uk). The Paterson Institute is located adjacent to The Christie NHS Foundation Trust, Europe's largest specialist cancer hospital. This provides exceptional opportunities for interactions between basic and clinical research teams and facilitates rapid translation of fundamental research findings into patient benefit. The University of Manchester has an ambitious vision for the future and is making a significant strategic investment in cancer and other areas by appointing new pioneering research staff through its World Leading Minds campaign (www.worldleadingminds.manchester.ac.uk/).

We seek four outstanding individuals to establish vigorous and ambitious independent research programmes. We are particularly eager to recruit to our priority areas, which currently include:

- Molecular pathology
- Mass spectrometry
- Lung cancer
- Melanoma immunology
- Pancreatic cancer
- Women's gynaecological cancers.

The positions are equivalent to Lecturer or Senior Lecturer and the successful applicants will have a PhD in a relevant area of cancer biology, appropriate post-doctoral training, and a proven track record demonstrated through high-impact publications. They will manage a group of research scientists and students and will be responsible for the professional development of their group members. Junior Group Leaders will be considered for promotion to Senior Group Leader posts at 6 years.

The generous package includes:

- Six years of core-funded support
- Competitive personal salary
- Up to three additional core-funded posts
- Up to two core-funded PhD students or clinical fellows
- Generous running expenses and start-up package for equipment
- High quality laboratory space
- Scope for group expansion through additional external funding
- Excellent research support facilities boosted by an £8.7m award from the UK Research Partnership Investment Fund (2012).

Informal enquiries can be addressed to the Director,
Prof Richard Marais (E: rmarais@picr.man.ac.uk).

To apply for these positions please send a CV, the names of three referees, a short summary of past research and a short research proposal. Also include the CV cover sheet and Equal Opportunities form available at www.paterson.man.ac.uk/Jobs/. Please submit your application to the HR Department (E: jobs@picr.man.ac.uk; T: +44 (0)161 446 3231).

The deadline for receipt of applications is 28th February 2013.

www.paterson.man.ac.uk

POSITIONS OPEN



FACULTY POSITION IN CANCER Biology and Cancer Prevention

The Susan Lehman Cullman Laboratory for Cancer Research in the Department of Chemical Biology, Ernest Mario School of Pharmacy, Rutgers, The State University of New Jersey is seeking an outstanding investigator for a tenure or tenure-track position. Applicants should hold a Ph.D. degree or equivalent and have a strong track record for research in the areas of mechanisms of cancer causation and prevention, as evidenced by independent, currently funded research (RO1 or equivalent), high-impact publications, and demonstrated leadership activities in the field. A strong commitment to teaching undergraduate, graduates, and postgraduate trainees will be expected. The successful candidate will also be a member of The Cancer Institute of New Jersey, the Center for Cancer Prevention Research, the Environmental and Occupational Health Sciences Institute, and join a large life sciences community with opportunities to participate in a variety of research and teaching programs. Please mail or electronically send curriculum vitae, brief research plan, current and past grant support, and the names and addresses of three references to: **Dr. Allan H. Conney, Chair of the Search Committee, Department of Chemical Biology, Ernest Mario School of Pharmacy, 164 Frelinghuysen Road, Piscataway, NJ 08854-8020, e-mail: aconney@pharmacy.rutgers.edu. An Affirmative Action/Equal Opportunity Employer.**

FACULTY POSITIONS UIC

Department of Microbiology and Immunology

The Department of Microbiology and Immunology in the College of Medicine at the University of Illinois at Chicago (UIC) is seeking to fill two tenured/tenure-track faculty positions at the level of **ASSISTANT, ASSOCIATE, or FULL PROFESSOR**. Candidates with microbiology or immunology research interests encompassing areas relevant to diabetes, obesity, stem cell biology, or regenerative medicine are encouraged to apply. Each successful faculty candidate is expected to maintain a vigorous independent research program and participate actively in the teaching, research, and graduate training programs in the department. Generous laboratory space and startup funds are available. Applicants are required to have a Ph.D., M.D., or equivalent doctorate level degree, and a proven track record in research as evidenced by consistent scholarly publications and current extramural peer reviewed grant funding.

UIC is the largest institution of higher learning in the Chicago area and is a major center for research and education. UIC's College of Medicine is part of the Illinois Medical District, the largest complex of medical centers in the United States. The Department of Microbiology and Immunology occupies over 20,000 square feet of renovated or new space in the new college of medicine research building. The faculty members in the department have active and interdisciplinary research programs in cellular and molecular immunology, microbial pathogenesis, host-pathogen interactions, virology, and structural biology. The department also maintains active collaborations with colleagues within the UIC Colleges of Dentistry, Pharmacy, and Liberal Arts and Sciences.

Applicants must apply online at **website: <https://jobs.uic.edu/>**. Required application materials are: current curriculum vitae, bibliography, statement of current research interests, and the names and contact information for three references. Review of applicants will begin February 5, 2013 and continue until the position is filled.

For more information about the Department of Microbiology and Immunology, please visit our **website: <http://www.uic.edu/depts/mcni/index.htm>**.

The University of Illinois at Chicago is an Affirmative Action/Equal Opportunity Employer.

POSITIONS OPEN

FACULTY POSITION in Molecular/Cellular Neuroscience The Ohio State University Wexner College of Medicine

The Departments of Neuroscience and Molecular and Cellular Biochemistry at The Ohio State University invite applications for a joint tenure-track faculty position in molecular/cellular neuroscience at the **ASSISTANT, ASSOCIATE, or FULL PROFESSOR** level. We seek an investigator with a productive, NIH-funded research program focused on the molecular and/or genetic mechanisms of nervous system disease. We are particularly interested in candidates studying neural circuits, motor behavior, nervous system development, and/or synaptic plasticity who will synergize with our existing zebrafish and neuromuscular disease research groups. We offer generous space and startup funds, zebrafish and mouse facilities, and the opportunity to join an outstanding research community with strengths in both basic and translational science at the OSU Wexner Medical Center. The successful candidate will have a joint appointment in the Department of Neuroscience (**website: <http://medicine.osu.edu/neuroscience>**) and Department of Molecular and Cellular Biochemistry (**website: <http://biomed.osu.edu/mcbiochem/>**).

Please submit a curriculum vitae and statement of research interests in PDF format to **Cheryl Yoder** at **e-mail: cheryl.yoder@osumc.edu** and arrange for three letters of reference addressed to **Randy J. Nelson**, Chair, Department of Neuroscience to be sent to the same e-mail address. Review of applications will begin 1 February and continue until the position is filled. *The Ohio State University has a strong commitment to diversity and actively encourages applications from candidates from individuals of underrepresented groups. The Ohio State University is an Equal Opportunity/Affirmative Action Employer.*

POSTDOCTORAL TEACHING FELLOW in Plant Ecology and Evolution

The Department of Ecology and Evolutionary Biology (EEB) at Tulane University seeks to fill the Koch-Richardson Postdoctoral Teaching Fellowship in Plant Ecology and Evolution (**website: <http://tulane.edu/sse/eebio/about/kochfellow.cfm>**).

The position is a two-year appointment with faculty status and a start date of July 1, 2013. The department aims to recruit an outstanding Ph.D. scientist who will merge excellence in teaching (60%), research (30%), and service (10%). Applicants are encouraged to identify a potential faculty collaborator in EEB, although those interested in botanical subjects not represented among EEB faculty will be given full consideration. Applicants should describe at least two botanical courses they would be able to teach, one at an undergraduate level, and the other at a graduate/advanced undergraduate level. An application (curriculum vitae, statement of research interests, and statement of teaching philosophy and interests), descriptions of the courses proposed to be taught, and three letters of recommendation focusing on both teaching and research excellence should be submitted electronically to the Search Committee (**e-mail: ecolevol@tulane.edu**). Please write "KOCH FELLOW" in the subject line. Application review will begin on February 15, 2013, and the position will remain open until filled.

Tulane University is an Equal Employment Opportunity/Affirmative Action/ADA Employer committed to excellence through diversity. All eligible candidates are invited to apply.

POSTDOCTORAL FELLOW POSITIONS at the Johns Hopkins University School of Medicine are available to study the molecular and cellular mechanisms underlying chronic pain. Strong background and experience in molecular biology, pain biology, or electrophysiology are required. Preference will be given to applicants seeking first postdoctoral position. Please electronically send curriculum vitae to **Dr. Yuan-Xiang Tao** at **e-mail: ytao1@jhmi.edu**. *Equal Opportunity Employer.*

POSITIONS OPEN

POSTDOCTORAL ASSOCIATES

The Institute of Marine and Coastal Sciences at Rutgers University is seeking Postdoctoral Associates in the areas of biological, chemical, geological, and physical oceanography. Prospective candidates should foster creative research avenues and interactions among existing research programs and faculty expertise. These fellowships are one-year renewable appointments. Applications are presently being accepted and evaluated on an ongoing basis. However, applications received by February 15, 2013 will receive the fullest attention. To apply, please electronically send curriculum vitae, statement of research interest, and names of three references to **Dr. Richard A. Lutz** (Director, Institute of Marine and Coastal Sciences), **e-mail: postdocsearch@marine.rutgers.edu** (please include "Postdoc" in the subject line). *Rutgers is an Equal Opportunity/Affirmative Action Employer.*

POSTDOCTORAL POSITIONS

Two postdoctoral positions are available to study the molecular mechanisms underlying neurodegeneration. Applicants must have a Ph.D. degree with research experience in signal transduction mechanisms and/or in vivo models of neurodegeneration. Please send curriculum vitae to: **Santosh R. D'Mello, Department Molecular & Cell Biology, University of Texas - Dallas, Richardson, TX 75080. E-mail: dmello@utdallas.edu**.

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